From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

EVALUATION OF PROTEIN AND BIOLOGICAL BIOMARKERS IN BREAST CANCER

Gustaf Rosin

Karolinska Institutet

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Evaluation of Protein and Biological Biomarkers in Breast Cancer

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av
Gustaf Rosin
Fil. mag

Huvudhandledare:
Docent Johan Hartman
Karolinska Institutet
Institutionen för Onkologi-Patologi

Bihandledare:
Professor Juha Kere
Karolinska Institutet
Institutionen för Biovetenskaper och Näringslära

Med. Dr Ulf Hannelius
Karolinska Institutet
Tidigare Institutionen för Medicinsk Epidemiologi och Biostatistik

Fakultetsopponent:
Professor Heikki Joensuu
University of Helsinki
Department of Oncology

Betygsnämnd:
Docent Lars-Arne Haldosén
Karolinska Institutet
Institutionen för Biovetenskaper och Näringslära

Professor Annika Lindblom
Karolinska Institutet
Institutionen för Molekylär Medicin och Kirurgi

Docent Henrik Lindman
Uppsala Universitet
Institutionen för Immunologi, Genetik och Patologi

Stockholm 2015

Den mätta dagen den är aldrig störst. Den bästa dagen är en dag av törst. Nog finns det mål och mening med vår färd – men det är vägen, som är mödan värd – Karin Boye

Till min familj och vänner

ABSTRACT

Breast cancer is the most common malignancy in women around the world. There have been great improvements in treating the disease and today between 80-90% of the women survive at least 5-years after their primary diagnosis. Still, due to the high incidence of the disease more than 450.000 women die of breast cancer each year worldwide. Much of the improvements in breast cancer survival can be explained by better knowledge of the development and progression of the disease, hence the treatments have become more effective. Yet, we still cannot explain why one patient relapses and dies whereas another patient, with seemingly similar tumor, survives. Thus, the identification of novel and evaluation of present breast cancer biomarkers are vital steps in the progression of improving the survival rate of breast cancer patients.

Estrogen receptor alpha (ERα) is expressed in around 70% of the tumors. ERα is a good prognostic and predictive biomarker since it can effectively be targeted by endocrine treatment. The gene Dyslexia 1 candidate 1 (*DYX1C1*) has been shown to be overexpressed in cancer and also to regulate and be regulated by estrogen and its receptors. We evaluated the expression of DYX1C1 mRNA and protein in three independent breast cancer cohorts and its association to known breast cancer biomarkers and survival **(study 1)**. We observed that DYX1C1 expression was positively associated with ERα expression and also functioned as a prognostic marker for improved survival.

Biomarker expression in breast cancer is usually examined using immunohistochemistry (IHC) on whole tumor sections. During the diagnostic process, cells from fine-needle aspirations are usually examined, sometimes also by immunocytochemistry (ICC). ICC is also useful in a metastatic setting, and it is therefore important that the results are concurrent with IHC evaluation. We retrospectively examined paired IHC and ICC evaluation of ERα, progesterone receptor (PR) and Ki67 **(study 2)**. We found that there were significant differences in the grading using IHC compared to ICC. Thus, to ensure the proper pathological diagnosis of metastatic lesions, comparisons and validation of these methods to detect biomarker should be performed.

Estrogen receptor beta 1 (ERβ1) is expressed in both normal mammary tissue and malignant breast tumors. *In vitro* data point towards a protective role of ERβ1 with lower proliferation and less invasiveness when overexpressed. However, *in vivo* data are so far inconclusive, where some previous studies have reported better a prognosis, while others have reported no association or even a worse prognosis. We examined ERα, ERβ1 and splice variant ERβcx in breast cancer patients with clinically negative lymph node status **(study 3)**. We found that ERβ1 was an independent marker of good prognosis. Interestingly it was expressed in patients of all grades and age groups, whereas ERα was commonly expressed in low-grade tumors of older patients. ERβcx did not show any prognostic association, nonetheless, high expression was coupled to risk of synchronous lymph node metastasis.

Breast cancer stem cells have been shown to be highly tumorigenic, thus functioning as a biomarker of poor prognosis. The origin of these cells is not completely understood. Either they derive from normal stem cells or they are transformed from differentiated cells of the bulk tumor. Through exome sequencing we found that isolated breast cancer stem cells and mixed cells from the bulk of the tumor did not differ genetically **(study 4)**. We showed that mutations were present in both stem cells and non-stem cells of the bulk tumor and at the same allele frequency. Our data supports a transformation of stem cells from differentiated cells.

In conclusion, the evaluation of new and existing cancer biomarker has the potential to generate novel hypothesis on tumor biology and reveal new targets for treatment.

SVENSK SAMMANFATTNING

Bröstcancer är den vanligaste cancerformen bland kvinnor, och ungefär en av nio kvinnor i Sverige drabbas någon gång under sin livstid. Antalet som insjuknar i bröstcancer ökar stadigt, men trots det så dör inte fler av sjukdomen, utan dödsfallen minskar snarare något. Anledningen till detta tros vara att fler tumörer upptäcks tidigt, t.ex. genom mammografiscreeningen, samt att behandlingsalternativen blivit bättre Ett sätt att förbättra behandlingsmöjligheterna är genom identifiering och evaluering av så kallade biomarkörer för bröstcancer. Biomarkörer kan bland annat vara proteiner, gener eller celltyper som förutsäga hur en kvinna kommer svara på en viss typ av behandling eller vilken prognos hon har att överleva sjukdomen.

Östrogenreceptor alfa (ERα) är en av de viktigaste biomarkörerna i bröstcancer och återfinns i ca 70 % av tumörerna. Den är både en markör för god överlevnad och för om cancern kommer att svara på anti-hormonell behandling. Genen Dyslexia 1 Candidate 1 (*DYX1C1)* kopplades först ihop med risken för att drabbas av dyslexi. Senare fann man även att genen uttrycktes i vissa cancertyper, såsom bröstcancer. I **studie 1** undersökte vi därför om DYX1C1 mRNA och protein var vanligare i olika typer av bröstcancer, samt om närvaron av DYX1C1 kunde förutspå chansen för patienten att överleva. Vi fann att DYX1C1 var vanligare i tumörer som även uttryckte ERα, samt att kvinnor som hade DYX1C1 proteinet i sina tumörer hade bättre chans att överleva än de som inte hade det.

Som ett steg i diagnostiken av bröstcancer gör man en finnålspunktion av tumören. Detta görs för att undersöka risken för att tumören är elakartad. Ibland så undersöker man även bland annat nivåerna av ERα, progesteronreceptorn (PR) och proteinet Ki67 ifrån punktionen, genom så kallad immunocytokemi (ICC). En liknande undersökning görs även på den utopererade tumören, men kallas då immunohistokemi (IHC). Det är IHC undersökningen som till stor del ger underlag för den fortsatta behandlingen efter operationen. I vissa fall då en tumör senare spridit sig till andra organ måste dessa analyser göras med ICC då man oftast inte opererar ut dottertumörerna. Det är därför viktigt att analyserna med IHC och ICC har stor överenstämmelse. I **studie 2** undersöktes detta genom att jämföra analyssvaren för ICC och IHC hos kvinnor som opererats för bröstcancer. Vi fann signifikanta skillnader mellan ICC- och IHC-analyserna för ERα, PR och Ki67. Detta talar för att ICC och IHC analyserna bör valideras mot varandra vid varje laboratorium för att försäkra att dottertumörerna inte får fel ERα, PR och Ki67 klassificering.

Länge hade man endast funnit en östrogenreceptor, den tidigare beskrivna ERα. År 1996 fann man ytterligare en östrogenreceptor vilken då namngavs till östrogenreceptor beta 1 (ERβ1). Denna finns uttryckt i både normal bröstvävnad samt i bröstcancer. Försök på bröstcancercellinjer har visat att ERβ1 troligen har en skyddande effekt genom att minska celldelningen och spridningsförmågan hos cellerna. Resultaten från studier på patientmaterial har dock varit blandade, där vissa har visat att närvaro av ERβ ger ökad överlevnad samtidigt som andra inte har funnit den kopplingen. I **studie 3** undersökte vi hur ERα, ERβ1 och en

variant av denna, ERβcx, var kopplade till andra biomarkörer och till patientöverlevnad. Vi fann att uttryck av ERβ1 i tumörerna var kopplat till bättre överlevnad och att ERβ1 fanns hos kvinnor i alla åldrar och oberoende av utmognadsgrad. Även ERα var kopplad till bättre överlevnad, medan ERβcx däremot, förutspådde risken för att drabbas av en lymfkörtelmetastas.

Bröstcancerstamceller har potential av att vara väldigt elakartade, där endast ett fåtal av dessa krävs för att bilda en tumör. Detta innebär att de kan anses vara en biomarkör för dålig prognos. Det är ej helt känt hur bröstcancerstamceller uppstår, men det finns två huvudsakliga hypoteser; antigen bildas de från friska stamceller, eller så tillbakabildas de från utmognade cancerceller. För att undersöka ursprunget av bröstcancerstamceller i **studie 4** så jämförde vi mutationerna i dessa celler mot mutationerna i resten av tumören genom sekvensering av DNA. Vi fann att samma mutationer fanns i både bröstcancerstamcellerna och i resten av tumören, samt att frekvensen av mutationerna var lika. Detta stödjer hypotesen att stamceller uppstår från utmognade celler.

Sammanfattningsvis så är identifiering och utvärderandet av biomarkörer ett viktigt steg i utvecklingen av nya hypoteser och framtida specifika mål för behandling.

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* Both authors contributed equally

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LIST OF ABBREVIATIONS

ADH	Atypical ductal hyperplasia
AF1	Activation function-1
AF2	Activation function-2
AI	Aromatase inhibitor
ALH	Atypical lobular hyperplasia
ALDH1	Aldehyde dehydrogenase 1
AREG	Amphiregulin
BMI	Body mass index
CSC	Cancer stem cells
DBD	DNA binding domain
DCIS	Ductal cancer in situ
DYX1C1	Dyslexia 1 candidate 1
E1	Estrone
E2	Estradiol
E3	Estriol
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
FNA	Fine needle aspiration
GH	Growth hormone
GWAS	Genome-wide association study
HRT	Hormone replacement therapy
HUT	Hyperplasia of usual type
ICC	Immunocytochemistry
IF	Immunofluorescence

IGF1	Insulin like growth factor 1
IHC	Immunohistochemistry
LBD	Ligand binding domain
LCIS	Lobular cancer in situ
MSC	Mammary stem cells
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PR	Progesterone receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
SERM	Selective estrogen receptor modulator
SLN	Sentinel lymph node
TDLU	Terminal ductal lobular unit
TGFβ	Transforming growth factor beta
TMA	Tissue microarray

1 INTRODUCTION

1.1 THE MAMMARY GLAND

1.1.1 Development and physiology

The mammary gland is a unique organ only present in mammals and sets us apart from other animals. It functions as a source of nutrition and energy for the offspring through production of breast milk. The breast tissue contains epithelial-, mesenchymal-, immune- and endothelial cells (1).

The development of the mammary gland starts during the embryogenesis and continues during adolescence, pregnancy and after menopause. The epithelial cells of the mammary gland have been hypothesized to have originated from apocrine sweat glands for more the 300 million years ago (2).

Much of the data on the developing breast have been obtained through mouse models, however, most stages and signaling pathways overlap between humans and mice (2). During the embryogenesis, cells from the ectoderm and mesoderm develop into the epithelial and stromal components of the mammary gland. At the moment, not all steps of the early embryonic development of human mammary gland are elucidated (2). There is little difference in the intrauterine mammary development between male and female fetuses (3). In mice, most of the signaling is done in para- or autocrine fashion. Key signaling molecules present are from the Wnt-signaling pathways and the fibroblast growth factor (FGF) pathways (2).

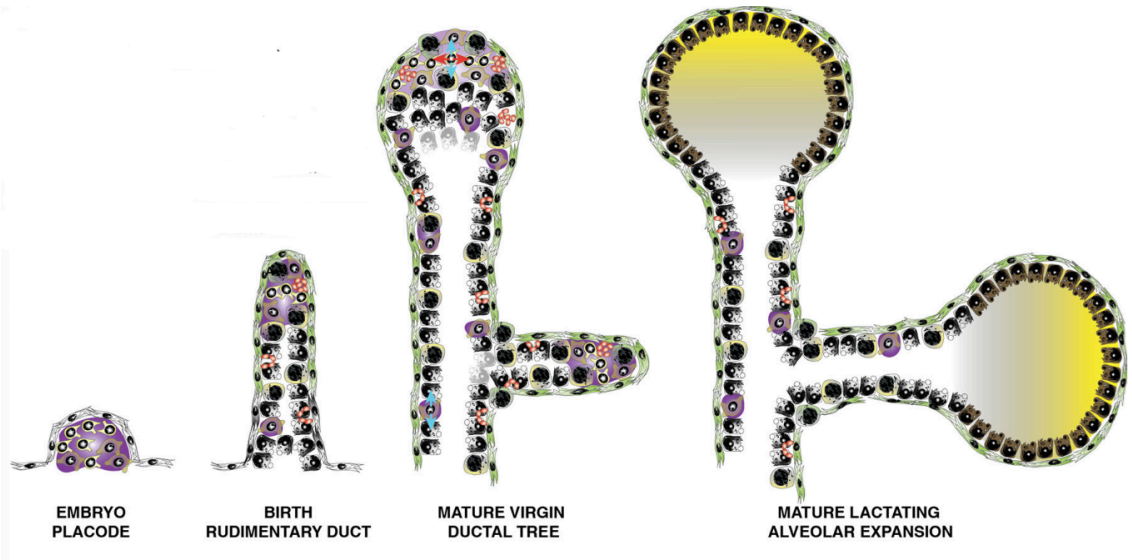

Figure 1. Schematic representation of the ductal and lobular development from embryo until mature lactating breast. Branching increases during the development. Modified from Oakes et al. 2014 (4). Reprinted with permission under the Creative Commons Attribution License.

The cells in this early stage of breast development will form a breast line and then migrate into the mesenchymal cells underneath and form what is known in mice as a placode (*figure 1*) (2). The placode will then form a breast bud, where in humans multiple sprouts are created

that will later unite at the nipple. The rudimental ductal units later elongate like the branches on a tree. After the branching of the ductal cells, a lumen will be formed. The mechanism is not clearly understood, but is believed to involve both apoptosis and autophagy (2). The epidermal layers of the skin will form the nipple through thickening and suppression of the formation hair follicles. The parathyroid hormone related protein and its receptor are thought to play a crucial role in the later stages of embryonic mammary gland development in mice (2). At birth the rudimentary mammary gland structure is in place for later maturation and development during puberty.

At puberty the hormonal changes present in the female body lead to development of both the stroma and the epithelium of the mammary gland. Proliferation of fibroblasts and adipocytes are central during this stage of the development (3). Elongation and further branching of the end bud structures, formed in the fetal development, into terminal ductal lobular units (TDLU) also take place. TDLUs are blunt ended acini are considered the functional unit of the mammary gland (3).

Several hormones are thought to play a vital role in the pubertal mammary gland development, among them human growth hormone (GH) and estrogen (*figure 2*) (2). GH stimulates the paracrine signaling of insulin like growth factor 1 (IGF1) from the stroma of the mammary gland. Knocking out either GH or IGF1 in mice, reduces the ductal formation of the mammary gland (5,6). Local IGF1 is thought to play a more important role than IGF1 produced in the liver (7). Interestingly high levels of serum IGF1 have been linked to increased risk of breast cancer (8).

Estrogen signaling and estrogen receptors (ER) are important in pubertal development. Their structure and signaling will be described in chapter 1.5 in more detail. Briefly, estrogen is a fat-soluble hormone, produced in the ovaries and the adipocytes (9). It exerts its effect by binding to the intracellular ER present in most tissues. There are two main ER subtypes; ERα and ERβ (10). Interestingly, during the mammary development ERα is not expressed in proliferating cells of the mammary tissue. The effect seems instead to be mediated mainly through paracrine signaling. Only a few cells with activated estrogen signaling are enough to drive the proliferation of the surrounding cells (11). This has also been shown in studies of ERα knockout mice, where the mammary development can be rescued by transplanting a few cells expressing ERα (12). The growth factors responsible for the paracrine signaling from the ERα expressing cells in the mammary gland are yet to be determined. However, several members of the epidermal growth factor (EGF) family such as Amphiregulin (AREG) have been suggested (2). AREG is believed to promote much of the proliferation seen by estrogen stimulation (13). It is strongly induced in mammary tissue during puberty, and knocking out Areg in mice leads to a phenotype similar to knocking out ERα. The AREG receptor is mainly located in the stromal cells and not in the epithelial cells of the mammary gland (14,15). The AREG signaling illustrates the complex and important cross talk between the stroma and epithelial cells in the mammary gland development in response to estrogens.

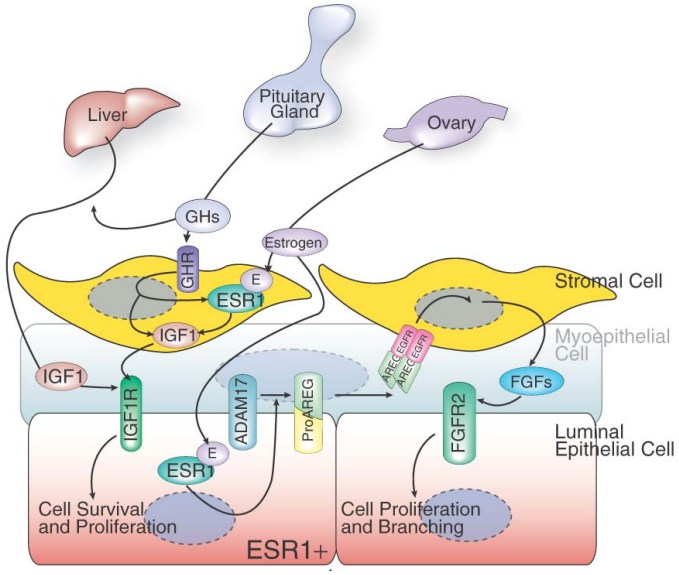

Figure 2. Schematic representation of the events, hormones and signaling molecules involved in the pubertal mammary development. Growth hormone (GH) released from the pituitary gland, stimulates the release of insulin like growth factor 1 (IGF1). Estrogen is released from the ovaries and binds to estrogen receptors (ESR). AREG and FGFs are important factors. For detailed explanation see the text. Modified from Macias and Hinck, 2012 (2). Reprinted with permission from John Wiley and Sons.

Fibroblast growth factors (FGF) are other candidates for the paracrine cross talk and are believed to mainly signal through one of its receptors, FGFR2. It is located on the epithelial cells and induce proliferation and elongation of the glandular ducts (16,17). On the other hand, transforming growth factor β1 (TGFβ1) has been shown to be the main negative regulator of branching and elongation of the ducts. Presence of TFGFβ1 leads to an increase of the space between the ducts (18-20). The inter-ductal space is later used for alveolar outgrowth during pregnancy, in the preparation of the breast for lactation. To summarize, a balance between promoting and inhibiting factors are important during puberty to develop a mature mammary gland with developed and dispersed ducts (*figure 3* displays the steps in mice mammary gland).

The adult female mammary gland goes through several changes during pregnancy. A combination of a large proliferative burst together with novel alveolar formation, known as alveologenesis, takes place before the birth of the child. Shortly after partum this is followed by milk production and start of lactation. Later, after weaning of the offspring, an involution of the mammary gland takes place. This leads to a restoration of the gland to most of its pre-pregnancy status (2). Again several hormones play a crucial part of the evolvement of the mammary gland of the pregnant female. The most important hormones are estrogen, progesterone, and the pituitary hormone prolactin (2). Due to ethical reasons, studies of healthy pregnant women are limited. Thus, data presented below have mostly been obtained from mouse models. Progesterone is similarly to estrogen a fat-soluble hormone with an intracellular receptor that function as a transcription factor (21). While progesterone does not seem to be obligate in the pubertal mammary development, it is vital for the branching and alveolar formation during pregnancy (22). Mice lacking progesterone receptor (PR) are

unable undergo alveologenesis. Overexpression of PR instead leads to increase in alveolar formation (23,24). PR is mostly expressed in the epithelial cells close to the lumen of the ducts. Although, the cells expressing PR do not seem to proliferate themselves, progesterone seems to promote proliferation in the mammary gland through paracrine signaling, similar to estrogen (25). The number of PR expressing epithelial cells decrease when mice become pregnant, from approximately 55% to 5% (23,26).

Progesterone and prolactin seem to have similar functionality and are closely related in the pregnant mammary gland (27). For example, when the MCF7 breast cancer cell line is treated with progesterone, the levels of prolactin receptor increase. The same happens for PR when treated with prolactin (28). The receptor activator NFκB ligand (RANKL) has been suggested to be responsible for some of the crosstalk between progesterone and prolactin (27). Furthermore, prolactin is important in the alveologenesis during pregnancy. Prolactin is also responsible for the continuation of lactation after partum and breast-feeding increases the release of the hormone from the pituitary gland (27).

The involution of the mammary gland happens upon weaning of the child and occurs in two steps (*figure 3*). Initially, there is massive apoptosis of the alveolar structures, mediated by local factors within the gland (3). Many of the mediating factors involved are known, however; the initiating trigger of apoptosis is unknown. The second irreversible step results in a dramatic remodeling of the gland. The end result is a structure more similar to that of the gland before pregnancy (2). However, the breast will not return completely to its pre-pregnancy state, since much of the branching of the ducts will remain. During menopause involution of the mammary gland also takes place. Leading to epithelial structures and inter-lobular connective tissue being replaced by adipocytes (1).

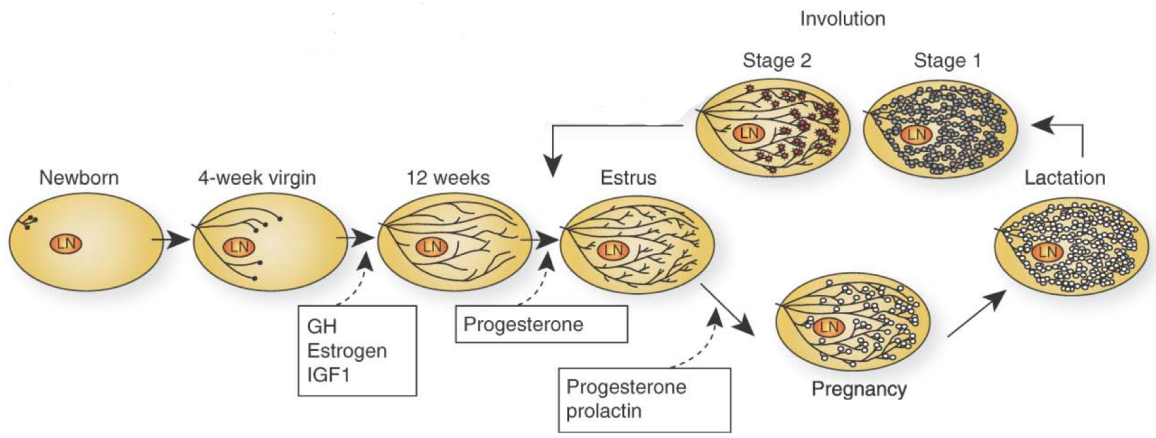

Figure 3. The development of the mammary gland in mice. From birth, adolescence, puberty, fertility, pregnancy, lactation and involution. Although the time points are different in humans, the overall development is similar as is the hormones responsible. Modified from Macias and Hinck, 2012 (2). Reprinted with permission from John Wiley and Sons.

1.1.2 Histology and anatomy

The female breast consists of many cell types, making up the epithelial and stromal compartment of the gland. The glandular part of the breast consists of epithelial cells that are

organized into ducts and lobes (*figure 4*). This is interspaced with connective tissue, made up of extracellular matrix and adipocytes (1,29). The fibrous connective tissue is organized into suspensory ligaments known as Coopers ligament. At the nipple, approximately 25 main ducts have their ending (1). The lobes are made up of smaller lobules that contain between 10-100 alveoli; this grouping is denoted as TDLU as described earlier. The alveoli are the location of the milk producing cells.

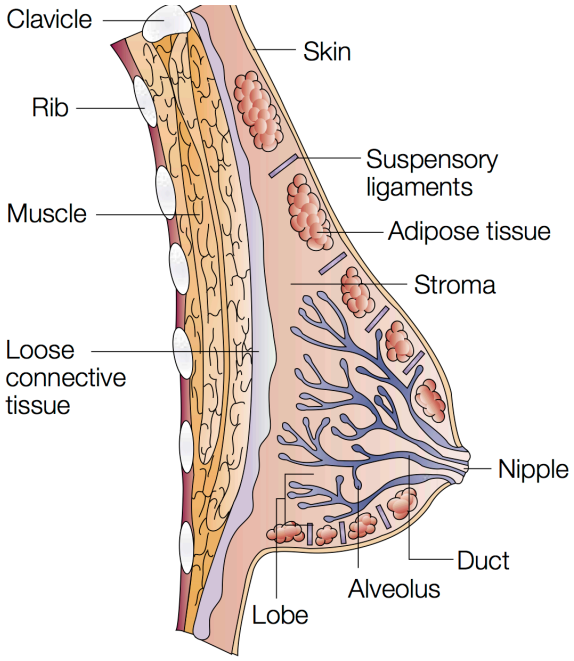

Figure 4. Schematic drawing on the anatomical structure of the mammary gland. Modified from Ali and Coombes, 2002 (30). Reprinted with permission from Nature Publishing Group.

The ducts and lobes of the human mammary gland epithelium are two cell layers deep. The inner cell layer, closest to the lumen, consists of cuboid epithelial cells known as luminal cells. These later become the milk producing cells during lactation (1). The outer layer is made up of myoepithelial cells. They surround the luminal cells and are believed to function similar to smooth muscle cells to propel the milk forward. Beneath the myoepithelial cells is the basal membrane, and also interspersed mammary stem cells that can mature into the two differentiated cell types (1). The stroma surrounds the epithelial compartment and is made up extracellular matrix protein such as collagens with scattered fibroblast, adipocytes and cells from the immune system.

The blood supply to the mammary gland comes from branches of the internal mammary artery and the lateral thoracic artery (1). During pregnancy and lactation, the blood flow increases to meet the subsequent increase in demand of nutrients and oxygen of the mammary tissue. The increased blood flow also transports immune cells into the gland and antibodies into the milk. The lymphatic system has received much attention due to its role in the spread of disseminated cells from breast cancer tumors (1). Most of the lymphatic fluid is drained to the axillary lymph node, both from the lateral and medial part of the breast. Whereas the deep parts are drained into internal lymph nodes of the breasts however, there is large individual variation (1).

1.2 BREAST CANCER

Breast cancer is the malignancy with highest incidence and deaths in women worldwide with 1.38 million new cases and 458.400 associated deaths in 2008 (31). Breast cancer affects women in both developed and developing countries *(figure 5)*. While, the incidence is higher in developed countries, the risk of dying of the disease is higher in developing countries (31). The difference in incidence between countries is partially explained by variations in the use of hormone replacement therapy and reproductive patterns, such as age at first child, number of children, age at menarche and nutritional factors (32). Furthermore, the variation in detection rate due to availability of mammography screening and medical care, also explain some of the differences (33). Other factors such as high alcohol intake, obesity and inactivity have also been linked to risk of developing breast cancer (34).

Figure 5. Incidence and mortality from breast cancer in 2008 of different regions of the world. Incidence (green bars) is highest in Europe, North America and Australia/New Zeeland. Mortality (red bars) is more equally distributed, highest rates are seen in Africa. Displayed as age standardized rates per 100.000. From Jemal et al. 2011 (31). Reprinted with permission from John Wiley and Sons.

1.2.1 Cancer development

The development of cancer is a multistep process. A normal cell can through several changes in its genome, both genetic and epigenetic, obtain abilities that are vital for a malignant cell. In a review from 2000, Hanahan and Weinberg introduced a simplistic cancer model with six hallmarks, all needed to form a malignant tumor from a normal cell (35). Later in 2011, two enabling and two emerging hallmarks were added to the six. The authors have been able to condense the vast scientific field of cancer into ten abilities, which are universal to all solid cancer tumors (36). The original six hallmarks were: "evading growth suppressors", "resisting cell death", "sustained proliferative signaling", "inducing angiogenesis", "replicative immortality", "tissue invasiveness and metastatic properties". The two new emerging hallmarks from 2011 were: "avoiding immune destruction" and "deregulating cellular energetics", and the two enabling: "genomic instability" and "tumor promoting inflammation" (*figure 6*).

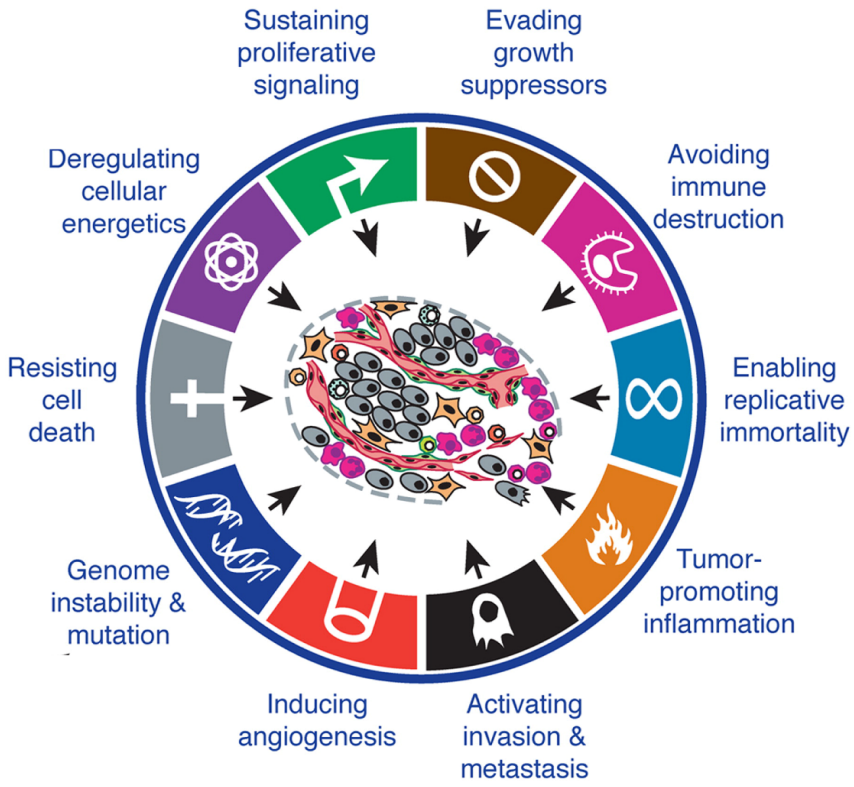

Figure 6. The ten hallmarks of cancer. The original six were presented by Hanahan and Weinberg in 2000. In 2011, two enabling and two emerging were added. Modified from Hanahan 2011 (36). Reprinted with permission under the Elsevier user license.

Different cancer types, but also different tumors from the same cancer type, acquire these hallmarks in different ways. The sustained proliferative signaling can be obtained by inducing growth factor receptor expression. For example in breast cancer, the *ERBB2* gene that encodes the HER2 receptor tyrosine kinase, is often amplified leading to overexpression and increased proliferation (37). Furthermore, signaling pathways can be activated or lose their normal inhibition. In breast cancer, the *PIK3CA* gene that transcribes the PI3K protein kinase is often constitutionally activated by mutations resulting in increased proliferation (38).

Evading growth suppressors is acquired mainly by loss-of-function mutation, deletions or downregulation of protein expression. The tumor suppressor gene *TP53* is commonly mutated which leads to inactivation of P53 and avoidance of apoptosis. This is especially common especially in more aggressive breast cancers (39). There several more ways by which cancer cells can evade apoptosis.

To obtain replicative immortality, the cancer cell needs to avoid the normal state of senescence, where the cell continues to exists without proliferating. Senescence is believed to occur when the telomeres of the chromosomes become too short (40). Overactivity of the telomerase enzyme, which lengthens telomeres, has been shown in several cancers including breast cancer. In normal epithelial cells, expression of the telomerase gene *TERT* is not detectable. On the other hand, in invasive breast cancer it is expressed in more than 90% of the tumors (40).

The rapid proliferation rate and high metabolic activity of cancer cells result in a high demand of nutrients and oxygen to the growing tumor. It has been suggested that tumors are unable to become larger than a few millimeters before their growth is inhibited by lack of these factors (41). Thus, the tumor has to be able to induce the formation of novel blood vessels, known as angiogenesis, from the surrounding normal tissue. This is considered to be an early event on the progression from pre-malignant to malignant tumors.

One of the most important differences between benign and malignant tumors is the potential of local tissue invasion and distant metastasis (42). The downregulation of cell-to-cell adhesion molecules is a common event in malignant tumor development (29). The ability of cancer cells to undergo epithelial-to-mesenchymal transition (EMT) is mediated through the re-activation of embryonic transcription factors and loss of adhesion. Resulting in a transformed epithelial cell that is able to survive despite the loss of cell-to-cell contact, invade nearby tissues, and later disseminate and migrate through blood or lymphatic vessels (43).

Genetic instability is one of the two novel enabling hallmarks (36). Having a genome where structural changes and mutations occur at a higher rate than in normal cells most often leads to loss of fitness for the individual cancer cell. However, this also leads to faster acquisition of novel abilities for some of the tumor cells, replacing the less fit cells. P53 plays an important role by inducing apoptosis if damages to the genome are found. Furthermore, *BRCA1* and *BRCA2* function as DNA repair genes to protect the stability of the genome. Having a germline mutation in either *BRCA1* or *BRCA2* results in a high risk of developing breast cancer (44).

The second enabling hallmark “tumor-promoting inflammation” and one of the emerging hallmarks “immune destruction evasion”, both involves the immune system in tumor development. When examining breast cancer sections histologically, many contain infiltrating immune cells. In immune deficient mice, the risk of developing tumors is increased. Humans undergoing immune suppressing treatment after organ transplantation have a higher chance of developing cancer derived from the donor organ (45). The immune

system is able to detect and eliminate many of the early stages of cancer. It has been estimated that as much as 80% of non-infectious cancers are eliminated before they can be detected (36). However, a tumor that has grown to a discoverable size has acquired the ability to avoid the immune system. In these tumors the presence of immune cells can instead promote the growth of the tumor. The release of growth factors from the immune system is believed to induce angiogenesis, proliferation and to modify the extracellular matrix in the tumor (36).

The second emerging hallmark is the ability to reprogram the energy metabolism of cancer cells. Many cancer cells are able to change their cellular metabolism from oxidative phosphorylation to rely heavily on glycolysis even though there still sufficient oxygen present (46). The reason for this switch is not clearly understood. It may intuitively seem unfavorable to rely on glycolysis since the energy produced per glucose molecule is much lower than for oxidative phosphorylation. It has been hypothesized that the production of other metabolic by-products needed for the fast replication of the genome may be the reason for the change in metabolic systems (46). A consequence of the decrease in oxidative phosphorylation would be lower oxygen consumption, but increase in glucose demand (36).

1.2.2 Breast cancer development

Breast cancer is believed to develop from the epithelial cells of the TDLUs in the mammary gland. The exact molecular steps of the progression from normal epithelium to invasive breast cancer are not completely understood. A breast tumor is considered malignant when it has invaded the surrounding tissue by passing through the underlying myoepithelial cells and basement membrane (47). To be able to transform from benign cancer in situ to invasive cancer, the myoepithelial cells need to lose their ability to contain the dysplastic cells. The mechanism of this is not clearly understood (48).

Several pre-malignant stages have been found in the mammary gland. These are ranging from hyperplasia, atypical hyperplasia, cancer in situ, to invasive cancer (47). The classical breast cancer development model is based on a stepwise progression from non- to pre- to malignant states, similar to the model proposed in colorectal cancer (48). This model was based on morphological studies of the changes found during histological examination. However, subsequent experimental studies have indicated that the process of breast cancer development is more complex (48). Many of the precursor stages may not be obligate, and not present in the development of all breast cancers. Also, not all pre-malignant lesions develop into cancer. Though, the presence of these indicates a higher risk of later invasive breast cancer development (47). Instead, it seems that tumors of different histological grades and subtypes have developed through different progression pathways (48). It has been shown in several studies that common losses and gains of chromosomal regions present in pre-malignant lesions are not present in all malignant tumors (48). Consequently, breast cancer today is not considered as one single disease but several malignancies originating from one organ (48).

Tumors have historically been classified according to histological type, where the two most common are ductal or lobular cancers, however many more exist. This division has shown some prognostic value (49). When examining the karyotype of breast cancer of different histological types, a overlap have been identified, thus indicating a non-perfect division (49). Counting the number of genetic aberrations in invasive lobular and ductal cancer, fewer are found in lobular cancer, which may reflect the average lower grade of lobular cancer (49).

One of the non-obligate benign precursors of ductal cancer is called hyperplasia of usual type (HUT) and show non-atypical intraductal growth. Only a few percent of the HUTs are believed to progress towards atypical ductal hyperplasia (ADH) and later ductal cancer in situ (DCIS) or to invasive cancer (48). HUT is instead mainly seen as a marker of risk for a female of developing invasive breast cancer. The benign histological precursor ADH, which has been seen as both a risk indicator and a non-obligate precursor, often shares many of the characteristics of low-grade DCIS. For example ERα and PR expression and lack of HER2 overexpression is common. Also the chromosomal aberrations are often the same between ADH and DCIS. Therefore there is no clear division between ADH and low-grade DCIS, instead a gradual shift takes place between the two (48). High grade DCIS is a non-obligate precursor of invasive ductal cancer and its presence is a strong predictor of later developing invasive breast cancer. High grade DCIS cells show high nuclear pleomorphism and necrosis is often present. However, since the cancer cells have not invaded through the underlying myoepithelial cells this state is considered benign. Most of the genetic and gene expression changes seen in invasive cancer can be found already in DCIS (48).

Lobular cancer has similar benign precursor stages as ductal cancer with atypical lobular hyperplasia (ALH) and lobular cancer in situ (LCIS). Both are risk indicators of developing invasive breast cancers as well as non-obligate precursor stages. The risk of developing invasive breast cancer is higher with LCIS than with ALH although the risk is low for both precursors. Analogous to ADH and DCIS, the division between ALH and low-grade LCIS has been considered to overlap by some (48). ALH have similar but more differentiated morphology compared to LCIS and the expression pattern of ERα, PR and HER2 show large similarities. High grade LCIS is often called pleomorphic lobular cancer in situ (PLCIS) and is considered a non-obligate precursor stage of invasive lobular cancer. PLCIS is characterized by pleomorphic, atypical nuclei, moderate proliferation and sometimes comedo-like necrosis. Differently from ALH and low-grade LCIS, the expression of ER and PR is usually low and HER2 is commonly overexpressed (48).

1.3 STEM CELLS

1.3.1 Stem cells in the healthy breast

Because of the cycling nature of the adult mammary gland it was long believed that there are cells within the gland with stem cell capabilities. These should be able to drive the proliferation needed during different stages in life during puberty, pregnancy, and menopause (50). In mice, it was early shown that by transplanting pieces of mammary tissue, to a mouse

without mammary gland, a whole gland could be regenerated (51). Later, a putative stem cell that was able to self-renew and reconstitute novel breast structures in mice was identified (50). The mammary stem cells (MSC) are believed to be present at only small numbers and give rise to both luminal and myoepithelial cells of the epithelium (4,52). Many MSC surface markers have been identified, such as Sca-1, CD24, CD49f, CD44, and ALDH1 (4,52). These can be used to both isolate and identify MSC.

Another way of identifying and isolating MSC is to make use of their capability to form free-floating cell aggregations, called mammospheres. When grown in serum free medium in non-adherent flasks the MSC aggregates together (53). The method of isolating and growing MCS was adopted from experiments from neurological stem cells (54). Epithelial cells that lose contact with either the extracellular matrix or other cells undergo apoptosis (55). This property is known as anoikis and exist to retain the organization of the epithelium and prevent dissemination of cells (56). Stem cells have the ability to prevent anoikis and survive in non-adherent conditions (57). Mammospheres are therefore highly enriched of cells with stem cell capabilities. It has been shown that a single cell from a mammosphere is able to form multilineage colonies when let to differentiate (53). MSCs are interesting for the development of cancer because of their longevity and ability to replicate many times, although the proliferation rate is low (52).

1.3.2 Stem cells in breast cancer

The existence and importance of stem cells in cancers were first described in leukemia. In leukemia only a subset of cells is able to reconstitute the disease after xenotransplantation (58,59). This discovery challenged the prevailing theory of the clonal evolution of cancer development, which states that any cell has equal probability of driving tumor development and proliferation (60). In breast cancer, the first potential cancer stem cells were described by Al-Hajj et al. in 2003 (61). They were identified as $CD44^{+}/CD24^{-/low}$ expressing cells and were over 50 times more tumorigenic than a mixed cell population to form new tumors (61). Therefore they can be considered as biomarkers of poor prognosis. Later, several other markers for identifying and isolating breast cancer stem cells (BSC) have been proposed, such as ALDH1, PKH26, DLL1 and DNER (4,62,63).

Mammosphere formation can also be used to isolate BSC (63). However, none of the suggested biomarkers seems to universally detect all stem cells, which remains one of the main controversies regarding the presence of BSC. The non-complete overlap results in that all $CD44^{+}/CD24^{-/low}$ cells do not express ALDH1 and there are a few cells within the mammospheres that are not $CD44^{+}/CD24^{-}$ or $ALDH1^{high}$. Critics against the presence of BSC have also argued that the $CD44^{+}/CD24^{-/low}$ cells may perhaps only be a subset of cells that are more suitable for xenografting, instead of being true stem cells (64).

There are differences in the prevalence of stem cells among the different intrinsic subtypes (described in chapter 1.4.8). For example the $CD44^{+}/CD24^{-/low}$ cells are more common in Basal subtype, whereas $ALDH1^{high}$ are more common in HER2 enriched tumors (65). Breast

cancer stem cells are also thought to be more common in the Claudin-low tumors than for example in Luminal A and Luminal B cancers (66). CD44 is the most commonly expressed stem cell marker in primary breast cancer with as much as 50% of the cells of the being positive. ALDH1 and CD24 are less common and expressed in fewer cells (65).

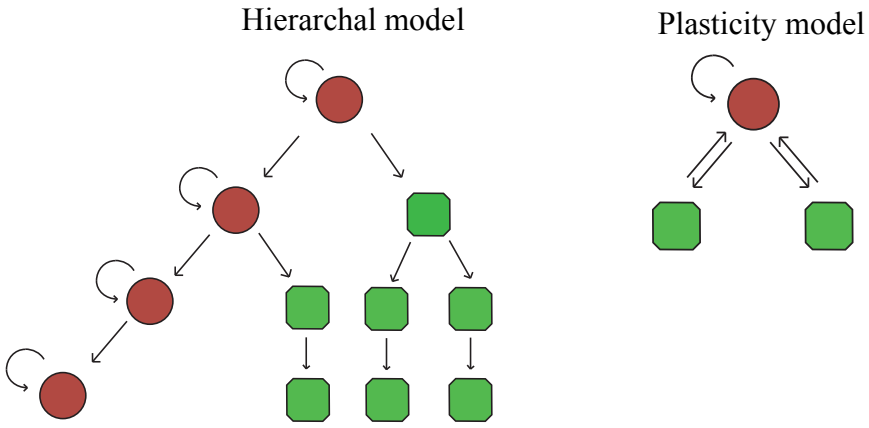

Figure 7. Schematic representation of the two main breast cancer stem cell models. According to the hierarchal model, cancer stem cells (red dots) they are able to renew and divide into more differentiated cancer cells (green squares). In the plasticity model differentiated cancer cells can revert back into cancer stem cells. Inspired by Gupta et al. 2011 (67).

1.3.3 Origin of stem cell in breast cancer

Several theories regarding the origin of BSC have been proposed. Two different main hypotheses are considered likely (*figure 7*) (68,69). The first, "Hierarchical division" suggests that cancer stem cells originate from the mammary stem cells of the normal gland (70). This means that several genetic modifications have taken place within the normal stem cell, resulting in it becoming malignant and causing the disease. The hierarchal model was the first hypothesis to receive support since it resembles how many normal stem cells are thought to behave. In this model one stem cell give rise, irreversibly, to the differentiated cells of the tumor. However, the model, has later received criticism since it cannot explain some of the characteristics seen in solid tumors (68,70). For example, the heterogeneity observed in tumors is difficult to explain with only one stem to cell give rise to the whole tumor. The presence of heterogeneity instead point towards a multiclonal evolution of the tumor with several subpopulations (70). Although stem cells have been found in normal mammary tissue, cancer stem cells do not have to originate from these. Their slow proliferation rate would make them less susceptible to transcriptional errors and subsequently mutations. On the other hand, their longevity makes it possible to accumulate mutations over time.

The second hypothesis proposes a stochastic plasticity model, where differentiated cancer cells have the potential to de-differentiate back into a more stem cell like state. These cells then drive the development and renewal of the tumor (64,71). The de-differentiation mechanism has been suggested to occur through changes in gene expression and mediate epithelial-to-mesenchymal transition (EMT) where epithelial cells transform into cell with

mesenchymal properties (72). EMT happens readily during the embryonic development and is also believed to be the mechanism to why some cancers metastasize (68). The hypothesis of breast cancer stem cell plasticity has the potential to unify clonal evolution theory with the stem cell theory, the two hypotheses of breast cancer origin (64). Recently, the Nobel Prize was awarded for showing that differentiated cells could be reprogrammed into pluripotent stem cells, so called iPS-cells. By inducing four different transcription factors cells could be de-differentiated into stem cells, this could plausibly also happen *in vivo* (73). Breast cancer cell lines can be stimulated to generate cells with stem cell like properties and express $CD44^{+}/CD24^{-/low}$ cells under certain conditions (74). The function and origin of cancer stem cells may be different in different cancers and more work is needed to fully understand the presence and role of stem cells in breast cancer. However, evidence is accumulating that cancer stem cells are cells that have acquired a stem cell like phenotype instead of being a group of cells with a specific genotype (67).

1.3.4 Cancer stem cells and therapy resistance

Studies have shown that cancer stem cells are less susceptible to conventional chemotherapy and radiotherapy (75,76), and several mechanisms have been proposed. For instance, up-regulation of detoxifying enzymes, efflux pumps, DNA-repair enzymes, and less response to apoptotic signals (77). This means that there is a need to develop novel drugs targeting this type of cancer cells (64). Several potential targets in breast cancer stem cells have been identified, among them Interleukin 8 receptor and DLL4 receptor, and intracellular enzymes part of the JAK/STAT pathway (62,69). Because of the possible ability of breast cancer cells switching between a differentiated and a stem cell like state, suggestions have also been made towards a combined therapy targeting the differentiated cells with conventional therapy and the stem cells with targeted therapy at the same time (78).

1.4 PROGNOSTIC AND PREDICTIVE FACTORS IN BREAST CANCER

1.4.1 Lymph node metastasis

The strongest prognostic factor in breast cancer is lymph nodes metastasis (79). The disseminated cancer cells from the tumor are most often transported by the lymphatic system. These cells can then settle the local or axillary lymph nodes, and form a lymph node metastasis. The lymph nodes have been proposed to function as filters where the cancer cells can be eliminated by the immune system, thus preventing spread to the systemic circulation and distant metastasis (47). A metastasis to the lymph nodes merits further surgical removal of all axillary lymph nodes. It often means that up to 20-30 lymph nodes will be removed. This procedure has shown to decrease the risk of local recurrence; however, if it protects against systemic metastasis is still not clear (80-82). Since lymph node metastasis is coupled to worse prognosis, these patients often require systemic chemotherapy and more extensive radiotherapy. Removal of the axillary lymph nodes sometimes leads to lymphedema of the arm, which is associated with reduced quality of life. Other side effects include neurological pain and limited shoulder and arm movement (83). To decrease the number of non-necessary

axillary dissections, the sentinel lymph node (SLN) biopsy surgical technique was developed (84,85). In clinically lymph node negative women, a blue dye and radioactive labeled fluid is injected in the breast prior to surgery. This helps the surgeon locate the first lymph node responsible for draining lymphatic fluid from the tumor, the so-called SLN. It has been shown that if the SLN is free from metastasis, this is associated with a low risk of spread to other lymph nodes, in some studies less than 10% (86,87). Therefore the benefit of removing all axillary lymph nodes in SLN negative patients does not outweigh the risk of developing adverse effects of the surgery. Furthermore, studies have been unable to show increased survival in node negative patients with extended axillary dissection (88).

1.4.2 Estrogen receptor alpha

ERα is one of the most important biomarkers, approximately 70% of all primary breast cancers are ERα positive. ERα is considered a good prognostic and predictive marker for endocrine treatment (89). In a study where no chemotherapy was given, the 5-year overall survival was 92% in ERα positive tumors compared to 82% in ERα negative tumors (90). However, evidence also point towards that ERα loses its prognostic potential with longer follow-up; after 5 years much of the difference is gone (91). Hence, it has been suggested that ERα expression denotes slower but similar potential of distant metastasis and death (79). The importance of ERα to predict response to anti-estrogen treatment is used clinically on a daily basis. There are three different classes of anti-estrogen treatments available with different modes of action; selective estrogen receptor modulators (SERMs) e.g. tamoxifen; aromatase inhibitors (AI); and the estrogen antagonist fulvestrant. Traditionally, a cut-off of 10% of positive cells has been used to separate positive from negative tumors. However, in 2010 the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) changed their guidelines and a new cut-off of 1% was implemented (92). The Swedish cut-off guideline is still at 10% positive cells. It has been shown that even patients with only little expression of ERα seem to benefit from endocrine treatment (93). In women with ERα positive tumors targeting ERα is effective, reducing the risk of recurrence by half for the first 5-years and by a third the following 5-years when given tamoxifen (94). It has also been shown that women with ERα negative tumors do not benefit from treatment with tamoxifen at all (94).

1.4.3 Progesterone receptor

Progesterone receptor (PR) is strongly associated with ERα expression and is measured as a marker of intact ERα signaling. It is therefore believed that PR expression better predict which patient who will respond to endocrine treatment (25,92,94). PR is a target gene of ERα activation. Treatment with estrogen leads to increased PR levels in breast cancer cell lines (95). Several estrogen receptor-binding sites, so called estrogen response elements (ERE), upstream of the PR gene, are believed to mediate the activation (96). The prognostic value of PR has been shown in several studies, even independent from ERα and other prognostic markers (97,98). There is today no cancer treatment that specifically targets PR.

1.4.4 Proliferation rate

The proliferation rate of the breast cancer cells is routinely measured by immunohistochemical staining of the Ki67 protein. Although its function is unknown, Ki67 is expressed in proliferating cells throughout the cell cycle (99). The Ki67 index is particularly important in the clinical decision making when determining between giving chemotherapy or not in ERα positive tumors. Thus, the Ki67 index can be used to discriminate between tumors with high or low risk of recurrence. But it can also be used as a proxy to discriminate between different intrinsic subtypes, such as tumors from the low proliferating Luminal A subtype with good prognosis, against Luminal B tumors with high proliferation and poor survival (100). However, there have been reports of variability in the reporting of Ki67 both between and within labs (100). Consequently, no general cut-off have been established to distinguish between tumors of high and low proliferation (101). There have also been discussions on how to analyze Ki67 most reliably in order to predict benefit of chemotherapy. Today most consider counting the percentage of Ki67 expressing cells within the areas of highest proliferation, the so-called hot spots, to be the most accurate (102).

1.4.5 HER2

HER2 is a biomarker that has evolved from a marker of poor prognostic into a predictive marker of treatment response (79,103). The protein is transcribed from the *ERBB2* gene located on chromosome 17. HER2 is a transmembrane receptor, which functions as a tyrosine kinase, although the endogenous ligand has not been discovered (104). Overexpression of HER2 was long seen as a poor prognostic marker until the development of the first targeted therapies. Without targeted treatment the patients have increased mortality and relapse rate (105). This is especially evident in node-negative patients (106). Today, using treatments targeting the HER2 receptor, the survival of these patients has improved dramatically (107). Early data described HER2 to be overexpressed in as high as 30% of tumors (108). However, due to better testing, the percentage of reported positive tumors has decreased to 15-20%, hence fewer false positive tumors are reported (109). To benefit from the anti-HER2 treatment the receptor needs to be overexpressed and also the gene amplified (104).

1.4.6 Staging

Staging of breast cancer patients reveals a great deal of information on the prognosis for the individual patient. In breast cancer, staging is performed according to the TNM classification system (110). This system is used in many cancers and divides the tumors into stage 0-4 depending on tumor progression. The factors taken into consideration are the size of the primary tumor (T), spread to loco-regional lymph nodes (N), and distant metastasis (M) (*table 1*) (110). Stage 0 is non-invasive cancer e.g. DCIS and LCIS. Stage 1-3 breast cancer (without distant metastasis) is today considered curable, while stage 4 breast cancer (with distant metastasis), is considered incurable. This is indicated by a meta-analysis on the prognosis from 36 clinical trials with metastatic disease showing a mean median overall survival of 21.7 months (111).

In node negative women, the size of the tumor is the most valuable independent prognostic factor (79). In women with tumors smaller than 1 cm, the 5-year overall survival has been reported to be as high as 99%. However, patients with 3-5 cm tumors had an overall survival of 86% (112). Furthermore, the mean time to distant metastasis was shorter for larger tumors compared to smaller tumors (113). The introduction of the mammography screening program has increased the number of early-detected tumors. This has led to that the average size of the tumors is now less than 2 cm (114).

Stage	Tumor (T)	Node status (N)	Metastasis (M)
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0-T1	N1mi	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1. Breast cancer staging. Tis = cancer in situ. T0 = no evidence of primary tumor. T1 = 0-2 cm. T2 = 2-5 cm. T3 = >5 cm. T4 = any size and growth to skin or chest wall. N0 = no lymph node metastasis. N1mi = metastasis between 0.2-2.0 mm. N1 = 1-3 axillary metastasis or 1 internal mammary node metastasis larger than 2.0 mm. N2 = 4-9 axillary lymph node metastasis. N3 = more than 10 node metastasis or metastasis to infra- or supraclavicular lymph nodes. N status can also be classified clinically. M0 = no distant metastasis or smaller than 0.2 mm. M1 = distant metastasis larger than 0.2 mm. Source UICC 7th edition TNM classification manual (110).

1.4.7 Histological grade

The differentiation grade of the tumor is used as a prognostic factor. There are several methods to evaluate the differentiation of the tumor. One of the most used and well validated is the Nottingham histological grading system (also called Elston-Ellis) (115). This grading system was developed from the Bloom-Richardson system by introducing numerical cut-offs for two of the three criteria (116,117). The criteria examined in the Nottingham grading system are tubular formation, nuclear pleomorphism, and mitotic count. Each is given a score between 1-3 which are then combined into a total score (116). The tumors are then divided into three separate grades; grade 1, tumors with a total score of 3-5; grade 2, total score of 6-7 and; grade 3, total score of 8-9 (116). As a group, grade 1 tumors have the best prognosis, and grade 3 have the worst (116,117). It has been shown that grade 2 tumors are a somewhat more common than grade 1 and grade 3 (117,118). However, the existence of grade 2 tumors has been debated. Some argue that tumors classified as grade 2 are in fact a mix of grade 1

and grade 3 tumors (119). The gene expression pattern of grade 2 tumors does not seem to be of a distinct type but instead match either grade 1 or grade 3 tumors (119). Also, analyses of chromosomal aberrations in different tumor grades have shown, contradictory to what was earlier believed, that progress from low- to high-grade tumors seldom happens. Common aberrations found within low-grade tumors are not found when examining high-grade tumors and vice versa (49). For example, loss of chromosome arm 16q and gain of 1q is common in low-grade tumors but is rare in high-grade tumors (48). This is especially evident in ERα positive tumors (48). High-grade tumors often have overexpression of HER2 but lack ERα and PR expression and have complex karyotypes with deletions and amplifications seen among many chromosomes (49).

1.4.8 Intrinsic subtypes

With the development of gene expression DNA microarrays, a novel way of classifying breast cancer was introduced in the early 2000s (120,121). By measuring the gene expression level of several thousands of genes in breast cancer tumors, a set of genes was identified that were differently expressed between tumors. Using this gene set, the tumors could be divided into distinct groups with similar gene expression pattern (121). The classification was called the intrinsic subtypes (molecular subtypes) and four principal subtypes were discovered (*table 2*). The main dividing factors in the clustering of the tumors were positive ERα expression status (120). Protein expression of keratin 8/18 was also common in this group. Since genes associated to the luminal cell type were overexpressed, the group was called the Luminal subtype (120). The Luminal tumors were later divided into two groups; Luminal A and Luminal B. The Luminal A subtype showed higher ERα expression and lower proliferation rate than Luminal B tumors (122). In the group of ERα negative tumors there was a group of tumors expressing genes common in myoepithelial cells, thus known as the Basal subtype (120). Another group of tumors within the ERα- negative group showed overexpression of HER2 and genes associated with HER2, thus called HER2-enriched (120). A final cluster of the ERα-negative tumors showed similarities to the expression of normal mammary specimens, and were then named Normal-like (120). The normal-like subtype has been questioned, as only being an indication of low amount of cancer tissue in some samples and hence showing a gene expression pattern similar to that of normal mammary tissue and immune cells. Therefore his group is usually not included among the main subtypes (122).

Luminal A	**Luminal B**	**HER2**	**Basal**
ERα+ and/or PR+	ERα+ and/or PR+	ERα-	ERα- and PR-
HER2 -	HER2+/-	HER2+	HER2-
Low Ki67	High Ki67	Usually high Ki67	Usually high Ki67

Table 2. Different tumor characteristics for estrogen receptor (ER), progesterone receptor (PR), HER2 and proliferation marker Ki67 within the established intrinsic subtypes. The Normal-like have been omitted. Adapted from Norum et al. 2014 (123).

The intrinsic subtypes have been linked to differences in incidence, survival rates and also response to treatment (121,122,124). Later studies have refined and shortened the list of genes in the subtype analysis. Today only 50 genes are necessary in the analysis, the so-called PAM50 classification (125). First in mouse models and then using larger groups of patients, less common subtypes such as the Claudin-low have been identified (66,123). The Claudin-low subtype seems to show similarities to mammary stem cells and be enriched of $CD44^{+}/CD24^{-}$ cells (126,127). Others have divided the Luminal subtypes into smaller sub-groups (122). The number of genes included in the subtype analysis is now small enough to be feasibly analyzed by qPCR instead of DNA microarrays. This opens up the possibility to use archived materials from FFPE samples and also to be performed at lower cost (125). Many studies have instead used IHC analysis on the routine pathological markers, ER, PR, Ki67, and HER2, as surrogate markers for gene expression analysis. This has been seen as a cheaper and more accessible method. Albeit, several studies have shown that these two different methods are not equivalent and many tumors are therefore classified differently (122).

1.5 ESTROGEN AND ESTROGEN RECEPTORS

1.5.1 Estrogen receptors

The estrogen receptors belong to the nuclear receptor superfamily, which consists of as many as 48 different members (128). Several of the nuclear receptors still have unknown function or unknown ligand; these are known as orphan receptors (129). The nuclear receptors show large structural similarities, with six distinct domains (129), indicating that the receptors have originated from a common ancestor and later evolved into several types with different physiological functions. Nuclear receptors are ligand activated transcription factors, meaning that when activated they alter in the transcription of genes (130). As previously briefly described, there are two main estrogen receptors subtypes (*figure 8*). The first one was discovered in the late 1950s, later named estrogen receptor alpha (ERα) (131), and the second in 1996, named estrogen receptor beta (ERβ) (132). Both ERα and ERβ are normally expressed in mammary tissue (133,134).

The gene for ERα, ESR1, is located on the large arm of chromosome 6 and gives rise to a full-length protein of 595 amino acids. There are several variants of ERα, resulting from alternative splicing of the transcript, the functions of these are less well understood than of the full-length protein (134).
The ERβ gene, ESR2, is located on chromosome 14 and encodes a 530 amino acid long full-length protein, known as ERβ1 (135). There are 5 known major transcriptional isoforms of ERβ, most of them with truncated C-terminal (10). The best studied is ERβ2, also known as ERβcx (136). ERβcx has no ability to bind ligand and therefore has no direct transcriptional activity. Instead, ERβcx is thought to mainly form heterodimers when expressed together with ERα leading to proteasomal degradation (137). However, ERβcx have also been shown to form dimers with ERβ1 (138).

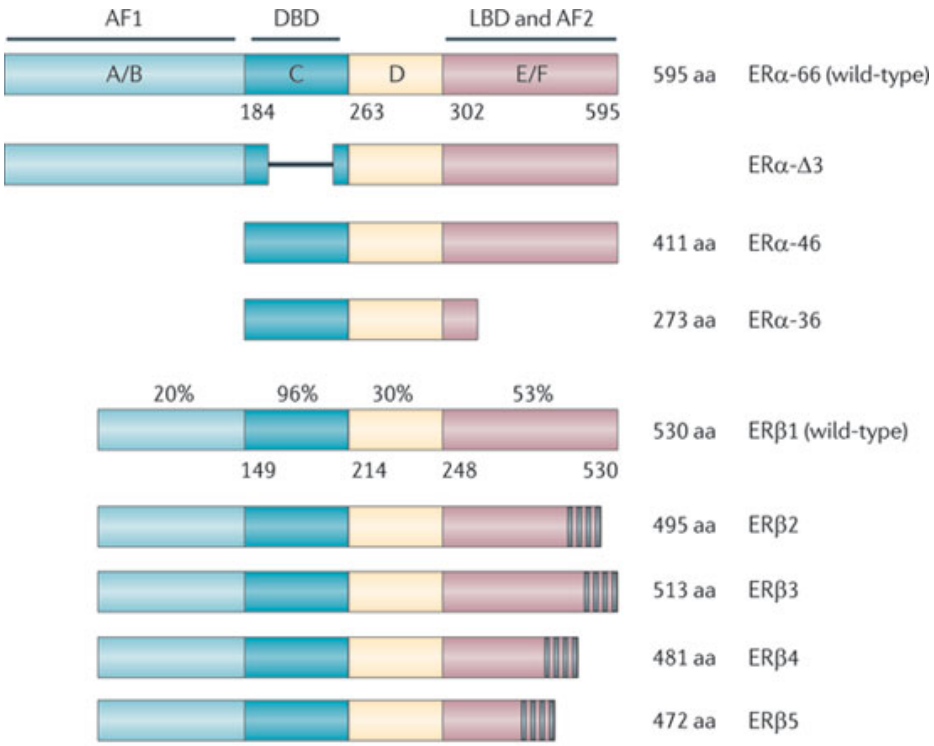

Figure 8. Schematic structure of ERα and ERβ and their splice variants. The domains are shown in different colors. Percentage denotes the homology between ERα wild-type and ERβ1. Amino acids (aa), activation function 1 (AF1), activation function 2 (AF2), DNA binding domain (DBD, and ligand binding domain (LBD). From Thomas and Gustafsson, 2011 (134). Reprinted with permission from Nature Publishing Group.

The overall protein structure is the same for ERα and ERβ. However, there are differences in the amino acid sequence resulting in, for example, differences in affinity for specific ligands (*figure 8*) (139). The six structural domains reflect the different functions of the receptor named A-F (140). At the N-terminus of the receptor (A/B domains) is the location of the ligand independent transcript activation function-1 (AF1) (141). This is also the domain with the greatest difference in amino acid sequence, with only 20% homology between ERα and ERβ (134). The DNA binding domain (DBD) identifies and bind to DNA sequences known as estrogen response elements (EREs) after ligand binding (142). In the DBD region there is also a dimerization box important for the dimerization of the receptors (142). The hinge region (D domain) is responsible for much of the structural flexibility, but also contain the nuclear localization signal (143). Closer to the C-terminus is the location of the ligand-binding domain (LBD) that also contains the ligand binging pocket (144). Next to the C-terminus is the ligand dependent transcript activation function-2 (AF2) (145), together with a second nuclear location signal (E/F domains) (146).

1.5.2 Estrogen signaling

The endogenous ligands of estrogen receptors are the female sex hormones. 17β-estradiol (E2) is the most prevalent in premenopausal women and is mainly produced in the ovaries (147). E2 has the same affinity for ERα and ERβ and causes transcriptional activation. Other estrogens such as Estrone (E1) are more predominant in postmenopausal women and Estriol

(E3) in pregnant women (148). The main mode of action is the same for the two ERs; after binding its ligand in the cytoplasm two receptors will dimerize and translocate into the nucleus where it will activate or repress the transcription of genes (134). Other modes of action have also been suggested such as activation by phosphorylation, described below (149,150). When ligands bind to the LBD, conformational changes in the structure of the receptors occur and facilitate dimerization (134). The receptors can be of either the same type, so called homodimerization (ERα/ERα or ERβ/ERβ), or of different types, known as heterodimerization (ERα/ERβ) (151). Different ligands lead to different conformational changes (152). This facilitates the recruitment of either co-activators or co-repressors, resulting in differences in the response. Depending on the availability of co-activators and co-repressors in different cells and tissues, the effect of the same ligands can differ (153). This has been shown in a tamoxifen resistance model where the co-repressors N-CoR and SMRT were downregulated and making the cells unable to be repressed by tamoxifen (154). On the other hand, co-activators of ERs are often over expressed in breast cancer (155).

Phosphorylation of ERα and ERβ has been shown to activate the receptors independent of estrogen binding. There are several known phosphorylation sites on ERα. For example, mTOR, MAPK and epidermal growth factor pathways, were able to phosphorylate ERα and lead to changes in the expression of ER target genes (156). This mechanism has also been linked to poor response of tamoxifen treatment and worse prognosis (149). Phosphorylation of ERβ is less well studied, although, in one study has suggested that phosphorylation is associated with better breast cancer prognosis (150).

1.5.3 ERα in breast cancer

For a long time ERα has been known to play a vital role in breast cancer. As early as 1896, Beatson could show that oophorectomy reduced the disease burden in young women with advanced breast cancer (157). Data from observational studies point towards lifetime estrogen exposure increasing the risk of developing breast cancer (158). For instance, early menarche and late menopause increase the risk of breast cancer (159). The increased risk is believed to mainly derive from an ERα mediated increase in proliferation and anti-apoptotic effects of estrogen on the mammary tissue (160). The use of hormone replacement therapy (HRT) have in large studies been associated with increased risk of developing breast cancer, both in randomized controlled trials and observational studies (161,162). The risk seemed to diminish after the treatment ended (162). However, using a different regimen (Estrogen alone) of HRT have yielded opposite results (161). Furthermore, HRT has been association with reduced risk of coronary heart disease when give to women with recent menopause (163). Today, it is considered that the benefit of symptom relief of HRT, given less than 5 years in women with recent menopause, outweighs the risk of breast cancer (164). Increased body mass index (BMI), which leads to higher serum estrogen levels, has been associated with increased risk of breast cancer in postmenopausal women (165). On the other hand, treatment with tamoxifen in women with ERα positive primary breast cancer leads to a decreased risk of developing contralateral breast cancer in the healthy breast (166). This

indicates that antagonizing ERα reduces the risk of developing breast cancer. However, this effect was not seen in women with ER negative primary tumors (166), indicating that estrogens are more important in some women for cancer development.

1.5.4 ERβ in breast cancer

Since the discovery of ERβ, its role in breast cancer has been under scrutiny and many studies have examined ERβ1 both *in vitro* and *in vivo* (167). For a long time, endogenous expression of ERβ1 was not believed to exist in breast cancer cell lines. However, recent studies have indicated the opposite, although generally the expression is low (168,169). Using overexpression in cell lines, ERβ1 has been shown to be anti-proliferative and function as a dominant negative regulator of ERα function (170-172). ERβ1 has also been suggested to have an anti-angiogenic role by decreasing the levels of PDGFβ (173). In addition a reduction of EMT and invasiveness have been shown, which was thought to occur through up-regulation of E-cadherin (174). In human tumor samples, ERβ1 and ERα show high degree of co-expression (175). ERβ1 is also less frequently expressed in invasive- compared to pre-invasive tumors (176,177).

Much of the *in vitro* data points towards ERβ1 having a protective role against breast cancer development, data from prognostic studies on patients show inconsistent results (167). Several studies have suggested an association of ERβ1 with favorable prognostic variables, such as longer disease free- and overall survival, smaller tumor size, fewer lymph node metastasis, lower grade, and improved tamoxifen response (178-182). Other studies have failed to show such a correlation (183,184). One study has even suggested an association between ERβ1 expression and worse outcome in node positive patients (185). The inconsistencies may be explained by a combination of factors, such as the use of different antibodies in different studies may result in differences in detection of one or several of the ERβ isoforms. Thus steps towards using well-validated commercial antibodies have been taken (186). Furthermore, the differences in grading systems could also affect the results (167). In addition, use of tissue micro arrays (TMA) in some studies could sometimes lead to loss of prognostic power. Mainly due to only a small area of the tumor being analyzed and therefore heterogeneous expression patterns may be missed. Expression of ERβ1 has been described in both the nucleus and the cytoplasm of breast cancer cells, subcellular localization have been taken into consideration by some of the studies but not all (182,187). The splice variant ERβcx is also commonly expressed in breast cancer tumors, however, it has been less well studied and its role is even less clearly understood than ERβ1(167).

1.6 DYSLEXIA 1 CANDIDATE 1 IN BREAST CANCER

Dyslexia 1 candidate 1 (*DYX1C1*) was the first candidate gene linked to the neurodevelopmental disorder dyslexia (188,189). DYX1C1 has been shown to regulate both ERα and ERβ1 through proteasomal degradation and form protein complexes together (190). Estrogen has been shown to also regulate DYX1C1 transcription (191). Other main functions

of DYX1C1 are believed to be in cellular migration and cilia formation and function (192-194). DYX1C1 overexpression has been shown in malignant breast tumors (195). Higher expression has also been shown in several invasive tumor types, including breast cancer, compared to normal tissue (196). Other genes associated to dyslexia, such as *DCDC2* and *ROBO1*, have also been linked with cancer. DCDC2 has been shown in prostate cancer and to be associated with poor prognosis and increased cell motility (197). ROBO1 has been coupled to increased migration in breast and several other cancers (198,199). In conclusion, the dyslexia candidate genes are an interesting group of genes with regard to their possible oncogenic potential, especially because of their role in cellular migration and ER regulation.

1.7 BREAST CANCER TREATMENT

While the incidence has increased in the Nordic countries during the last century, mainly due to changes in reproductive patterns and nutrition (200), the risk of dying when diagnosed with breast cancer has slowly decreased (*figure 9*) (200). Today the 5-year survival is almost 90% in Sweden in some age groups, mainly due to improvements in early detection and adjuvant treatment (200,201). There are five different categories of treatment; surgery, chemo-, radio-, endocrine- and targeted therapy (202).

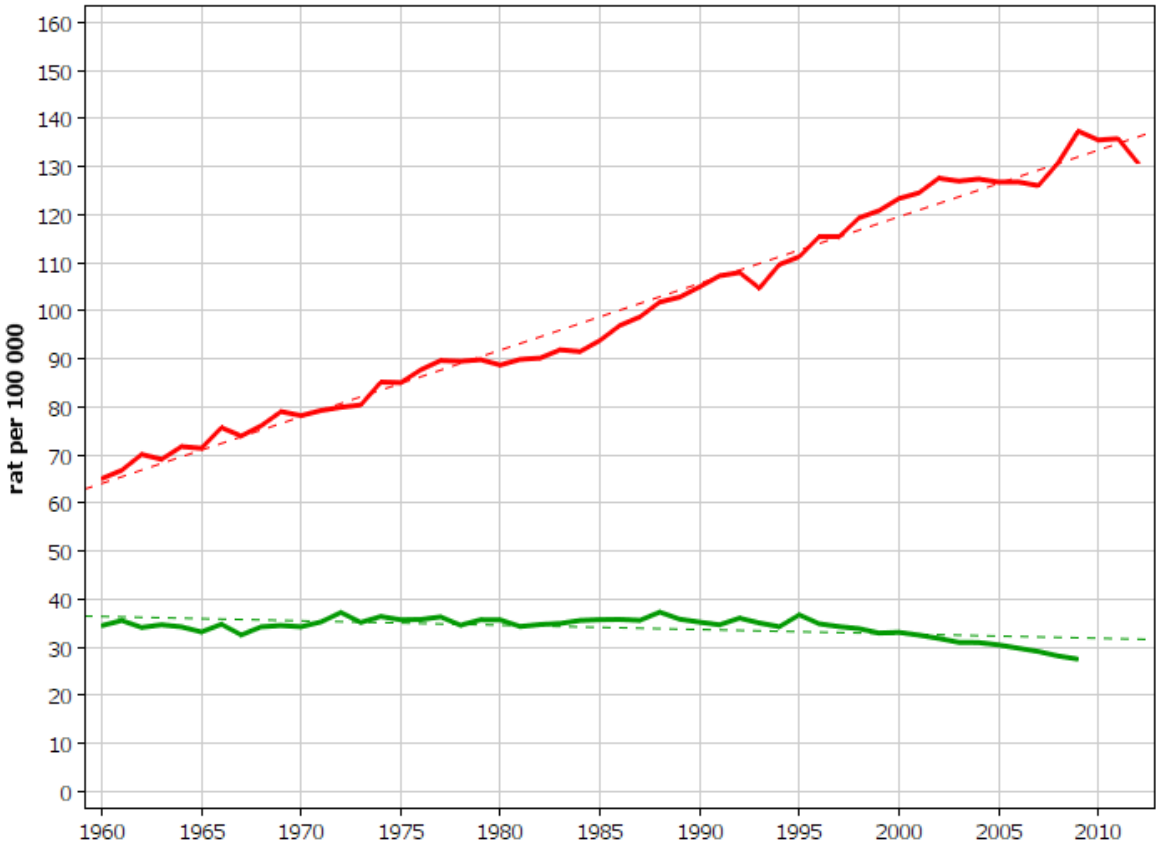

Figure 9. Incidence and mortality in the Nordic countries. The incidence of breast cancer has steadily risen (red solid line, dotted line represents trend). The mortality has slowly decreased (green solid line, dotted line represents trend). Age standardized rate per 100000 persons per year for the Nordic countries. Standard age structure from 2000 in the Nordic countries. From NORCAN (203).

1.7.1 Surgery

Surgery is considered the main pillar of breast cancer treatment and cures a majority of the patients. On small tumors, both total and partial mastectomy surgical techniques are considered equally efficient in preventing local recurrences and distant metastasis, when combined with radiotherapy (204,205). Presently, the sentinel lymph node (SLN) biopsy surgical technique is used routinely in women with clinically negative lymph node status (88). It is considered effective in staging the patients, with less co-morbidity than axillar dissection (87,206). The sensitivity of finding lymph node metastasis using frozen sectioning and staining during surgery is 75-80% (207).

1.7.2 Radiotherapy

A meta-analysis of postoperative radiotherapy has shown that it reduces the risk of local relapses and improves the survival of the patients undergoing either breast preserving surgery or total mastectomy (208,209). The benefit of radiotherapy is most evident in women with higher risk of recurrence, specified as a recurrence risk of 20% over 10 years (210). However, radiotherapy is associated with increased morbidity, such as reduced lung function, lymph-edema of the arm, and rashes to the breast and skin (211,212). Radiotherapy is given to breast cancer patients in repeated small doses to reduce these side effects (213).

1.7.3 Endocrine treatment

Endocrine treatment antagonizing ERα or the production of estrogens is recommended to all women with ERα positive tumors (202). Tamoxifen functions through selective modulation of ER activation, in breast tissue it works as a partial antagonist (214). Treatment with tamoxifen is effective in reducing the risk of recurrence (215). It also improves the overall survival by almost 50% and breast cancer specific survival by one third during the first 5 years (94). Prolonging the treatment to 10 years has shown even better results (94).

Aromatase inhibitors (AI), block the conversion of androgens into estrogens by the aromatase enzyme in tissues where it is expressed (216,217). Aromatase has been shown to be upregulated in breast cancer (218). The use of AI is slightly more efficient than tamoxifen in preventing recurrences (219). However, AI is only used in postmenopausal women since it, because of biological feedback, does not inhibit estradiol production in the ovaries in premenopausal women (220). The side effects of AI is coupled to higher risk of osteoporosis than tamoxifen, but lower risk of thromboembolic events (221).

Some trials have used sequential treatment of first tamoxifen and then AI or vice versa. Studies are still ongoing and the optimal order and duration for endocrine treatment is still not known (202). Although the anti-estrogen treatment is efficient in many women, some develop relapse during ongoing tamoxifen treatment. Several mechanisms have been proposed (30). One is believed to be phosphorylation of ERα resulting in ligand independent activation (156). Another mechanism for acquiring resistance has been through downregulation of ERα leading to loss of estrogen dependence (222), this is thought to occur

in 20% of the tumors (223). Also changes in cofactor levels have been shown (224). Increased expression of the truncated splice variant ERα-36, which has been shown to be located on the plasma membrane and in the cytoplasm, and be associated with tamoxifen resistance (225). Gain of function mutations discovered in ERα, leading to constitutional activation, has also been involved in the resistance to anti-estrogen treatment (226).

Other challenges regarding the use of anti-estrogen treatment is to identify the patients with ERα positive tumors that will not benefit from the treatment. Methods of pre-evaluating the benefit of receiving chemotherapy have been developed. The so-called 21-gene recurrence score is one of these (227). Identifying similar recurrence score for giving anti-estrogen treatment or not would be beneficial to many women. Although endocrine treatment is efficient, a obstacle is treatment compliance. As many as 31% of the women did not follow the treatment recommendation (228). The main reasons are usually due to of adverse side effects (229).

1.7.4 Chemotherapy

Cytostatic drugs are only given to women with high risk of relapse, mainly because of the risk of severe somatic side effects, such as bone marrow suppression (230). Also the socio-psychological side effects, for example hair loss, can be very burdensome (230). Chemotherapy efficiently reduce the risk of mortality and are usually given in combinations of several drugs (231). There are several types of chemotherapy, the most common combinations in breast cancer are CMF (cyclophosphamide, methotrexate and fluorouracil), FEC (epirubicin, cyclophosphamide and fluorouracil), FAC (fluorouracil, doxorubicin (adriamycin) and cyclophosphamide), AC (doxorubicin (adriamycin) and cyclophosphamide) and EC (epirubicin and cyclophosphamide) (232). These target vital elements in dividing cells thus inhibiting proliferation and leading to apoptosis, therefore they are more effective in highly proliferating tumors (233). The relative risk reduction when giving chemotherapy has been reported between 22-36% depending on the drugs used and the severity of the patients disease (232).

Neoadjuvant treatment using chemotherapy, together or without anti-HER2 treatment, is less common in Sweden and given mostly to patients with locally advanced tumors (202). However, neoadjuvant chemotherapy has in resent years been shown to be as efficient as adjuvant treatment on large tumors, therefore the use may further increase (234-236).

1.7.5 Targeted therapy

Targeted therapies are the most recent addition to the treatment options available in breast cancer. In the middle of the 1990's the monoclonal antibody trastuzumab, targeting the HER2 receptor, became available (237). Trastuzumab is a monoclonal antibody that binds to HER2 present on the cell membrane. Several mechanisms of actions have been shown. Among them downregulation and internalization of HER2, increased antigen-dependent cellular cytotoxicity, cell cycle arrest, induction of apoptosis, and reduced angiogenesis (238,239).

When used, mortality has been shown to be reduced in women with amplified HER2, in both metastatic and primary disease (240,241). Given together with chemotherapy it reduced the relative risk of mortality with as much as 34% (242). However, some women develop heart failure as a side effect of the treatment, thus the function of the left ventricle is examined both before and during the treatment with trastuzumab (242). Other drugs targeting HER2 have more recently become available, for example pertuzumab which inhibits receptor dimerization (243). HER2 overexpression can also be targeted by the small molecule lapatinib which inhibits the tyrosine kinase function of the receptor (244).

Other targeted therapies, such as anti-angiogenic antibodies targeting VEGF, have shown to be efficient in advanced lung and colon cancer (245,246). However, no difference in recurrence rate or survival was seen when used together with chemotherapy in breast cancer negative for ERα, PR and HER2 (247). In ERα positive tumors, inhibition of mTOR using everolimus has shown promising results (248). The mTOR/Akt/PI3K pathway has been implicated in resistance to tamoxifen treatment (249). Thus, inhibiting mTOR, which is a downstream enzyme in the pathway has induced apoptosis in cells deprived of estrogen (250).

1.8 BREAST CANCER HEREDITY

Most breast cancers are considered to be sporadic. These arise because of stochastic events during cellular replication, where no specific genetic or environmental cause or risk factor is known (251). Nevertheless, there is a hereditary component in breast cancer. Both polygenetic risk alleles with slight increases in the risk, and monogenetic hereditary breast cancer syndromes have been discovered (252).

Genes associated with risk of developing breast cancers can be divided into high, moderate and low penetrance genes. The high penetrance genes are rare in the population while low penetrance genes are common (252). In total, the high penetrance genes are believed to be responsible for around 25% of all hereditary breast cancer, meaning that the rest is caused by medium and low penetrance genes (253).

1.8.1 High and medium penetrance genes

There are several identified high penetrance genes known to cause breast cancer; the two most prevalent are *BRCA1* and *BRCA2*. They are believed to cause around 5% of all breast cancer cases and around 15 % of all hereditary breast cancers (254). Other genes responsible for familiar breast cancer, such as *PTEN*, *TP53* and *CDH1*, are both less common in the population and have lower penetrance compared to *BRCA1* and *BRCA2* (252). A meta-analyses has reported a cumulative risk of 57% for *BRAC1* carriers and 49% for *BRCA2* carriers of developing breast cancer at the age of 70 (255). Many women who are known carriers therefore undergo prophylactic mastectomy, which has been shown to reduce the risk of developing breast cancer (256).

There is also a group of medium penetrance genes that approximately doubles the lifetime risk of developing breast cancers (252). Because they are relatively rare and have only moderate increase in risk, they are less clinically relevant. It has been estimated that these genes contribute with less than 3% of the hereditary relative risk (257). However, it is likely that more medium penetrance genes will be discovered (252).

1.8.2 Low penetrance genes

Low penetrance genes, which are common in the population, have mainly been discovered through genome wide association studies (GWAS) (252,258). Approximately 30 GWAS have been carried out on breast cancers patients (259). After the latest large GWAS, which was published in 2013, 41 novel loci were added to the 27 loci that had already been identified (260). Together they explain only a total of 14% of the hereditary risk of breast cancer, since each locus only contributes with a small increase in risk (260). Of the novel loci discovered the highest odds ratio was 1.26 and together the 41 new loci explained only 5% of hereditary risk. The authors therefore concluded that there are several thousands of undiscovered loci that contribute to the hereditary risk in breast cancer (260). Much of the unexplained hereditary risk is thought to be explained these by low penetrance genes (261), together with additional low frequent high penetrance genes, structural differences, gene-gene interactions and gene-environment interactions (262).

For most of the risk loci the mechanisms of the increased risk are unknown (263). However, there are some exceptions e.g. FGFR2 and MAP-kinase. FGFR2 is a member of the tyrosine kinase receptor family, which induces cell growth, proliferation, angiogenesis and cell motility (264). The MAP3K1 gene that encodes the MAP-kinase protein is important in the MAP-kinase signaling pathway and is associated with HER2 signaling (264).

2 AIMS OF THE THESIS

The general aim of the thesis was to find and evaluate novel and recently discovered breast cancer biomarkers to use for prognostication and later potential targets for treatment, focusing on estrogen receptors, DYX1C1 and stem cells.

Aim of paper I - Investigate the dyslexia susceptibility gene DYX1C1 as a potential breast cancer biomarker and its role as a prognostic biomarker in women diagnosed with local breast cancer.

Aim of paper II – evaluate the correlation of immunocytochemistry and immunohistochemistry on expression pattern of ERα, progesterone receptor and Ki67 in fine-needle aspirations and surgical resections from the same patients.

Aim of paper III – examine the prognostic role of ERβ1 and ERβcx (ERβ2) in patients with primary breast cancer who had undergone sentinel lymph node biopsy surgery.

Aim of paper IV – examine the mutational spectra of breast cancer stem cells compared to the bulk tumor/non-stem cells, using next generation sequencing to elucidate the origin of breast cancer stem cells.

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS

3.1.1 Uppsala cohort

The Uppsala breast cancer cohort, used in study 1, consists of altogether 315 patients diagnosed with invasive breast cancer in Uppsala County between 1987 and 1989 (265). This represents 65% of all patients diagnosed with breast cancer at that time period in Uppsala. The clinical data and pathological characteristics of the tumors were collected from the patients' records (265). Using registries, follow up has been updated several times by examining the survival status of the patients together with the cause of death. Global gene expression analysis was performed using Affymetrix microarray chips on 260 of the patients within the cohort. The analysis was performed on all the patients which had sufficient and high enough quality mRNA(265). The tumors were then classified into the intrinsic subtypes described earlier. Due to the construction of TMAs from the original formalin fixed paraffin embedded (FFPE) tumor tissues, the cohort can still today be used to examine the expression levels of potential novel cancer biomarkers using immunohistochemistry (IHC) techniques.

3.1.2 Stockholm cohort

The Stockholm breast cancer cohort, used in study 1, consists of altogether 524 patients with surgically removed invasive breast cancer (266). The inclusion dates were January 1st 1994 to December 31st 1996. 280 patients had available tumor tissue. Clinical and pathological data for tumor size, lymph node status, hormone receptor status, treatment, date and site of relapse and cause of death, were collected from the Stockholm-Gotland breast cancer registry (266). The histological grade was re-examined by an experienced pathologist. Of 280 tumors, 159 were examined using global gene expression microarray chips from Affymetrix. Reasons for exclusion, from the gene expression analysis were lack of available frozen tumor tissue, emigration abroad or refused to participate, low quality or amount of the extracted RNA, or that the patient had received neoadjuvant therapy (266). The patients excluded because of lack of frozen tissue had on average smaller tumor size, fewer affected lymph nodes and less recurrences. However, the patients excluded due to other reasons did not differ from the patients included in on the microarray analysis (266).

3.1.3 CHARES cohort

The CHARES cohort, used in study 1, is a population-based case-control study of 3345 women diagnosed in with invasive breast cancer between October 1st 1993 and March 31st 1995 in Sweden. Controls were frequency-matched to the age structure of the cases (267). Out of these women, 61 cases were randomly selected for RNA extraction and PCR analysis. 77% of the selected cases were positive for ERα expression.

3.1.4 Immunochemistry concordance cohort

The immunochemistry concordance cohort, used in study 2, consists of all patients diagnosed with invasive ductal or lobular breast cancer at Karolinska university hospital during 2011. Altogether 454 patients were identified. Patients who had received neoadjuvant chemotherapy, had not undergone fine-needle aspiration (FNA) or had longer then 100 days between FNA and surgical removal were excluded, resulting in 346 patients. Pathological data was retrospectively extracted from the patient's medical records. However, biomarker analysis was not performed on FNA in all patients, thus data on for ERα was available on 133 patients, PR on 80 patients, and Ki67 on 131 patients.

3.1.5 Sentinel node cohort

The Sentinel node patient cohort, used in study 3, was expanded from a part of a large study with the aim to investigate the efficacy and safety when introducing the sentinel lymph node biopsy technique in Sweden (268). Patients operated using the sentinel lymph node biopsy technique in Stockholm between January 1st 2001 and December 31st 2006, with available tumor tissue, were included. In the end the cohort consisted of 340 patients. All included patients had clinically negative axilla. About half of them had lymph node metastasis. These were either discovered by the pathologist using frozen sections or later after paraffin embedding and cytokeratin staining. Clinicopathological characteristics, such as size of the tumor, treatment, histological grade, HER2 overexpression, time of follow-up, recurrences and cause of death, were collected from the patient records.

3.2 BIOLOGICAL SAMPLES

3.2.1 Formalin-fixed paraffin embedded tumor tissues

An invaluable source of materials in the field of oncology comes from the archives of formalin fixed paraffin embedded (FFPE) tissue blocks. After the surgical removal of a breast tumor, the tissue is then placed in 4% formaldehyde for up to 72 hours. It is then cut into smaller pieces and embedded in paraffin. The paraffin embedded tissue blocks can be cut into thin sections and mounted on a glass slide. These are later stained and used in the routine pathological examination. Most clinical pathology units save the remaining FFPE blocks after the routine clinical examinations to be able to re-analyze or perform new staining in the case of a recurrence. In addition, many of these FFPE collections are often available for research purpose. Commonly they are used to examine the expression of proteins within the tumor using methods such as immunohistochemistry (IHC) or immunofluorescence (IF). The development of next generation sequencing (NGS) techniques and refinement of global gene expression arrays have opened up these archives of tumors to be examined using modern DNA sequencing and RNA expression analysis. The benefits using these FFPE collections are the long follow-up time and large number of tumors available, while the drawbacks are the lower quality of the RNA and DNA that can be extracted from the FFPE samples.

3.2.2 Scrapings and primary cultures

Snap fresh-frozen pieces of tumor tissue are the most widely used method for research biobanking today. It is suitable technique to obtain high quality tumor material for later RNA and DNA extraction. However, there are several problems with the method. For example, there is a risk of potentially removing a section from the tumor that will not be analyzed during pathological examination. This may in the worst case lead to that the wrong diagnosis or treatment is given. This is especially evident in small tumors where the removed piece for biobanking is relatively large to that of a bigger tumor. This results in that many of the smaller tumors are not included in the biobank, thus introducing selection bias (269).

We have introduced a novel method of collecting tumor cells for biobanking without the need to remove a piece of the tumor (269). Cutting the tumor in half and using a scalpel to scrape 3-10 times around the surface of the tumor cells can be collected. The yield is enough for several cell- and molecular biology methods such as RT-qPCR of mRNA, FACS sorting, IF and cytological staining, DNA extraction for pyrosequencing, and cells for cultivation. The material from the scrapings can be stored in liquid nitrogen or be used for analysis directly. This method has the potential to increase the number of patients with tumors suitable for biobanking. We have estimated that the inclusion of patients could increase from around 60% to 85%, with little increase in workload for the pathologist (269).

Breast cancer stem cells can be enriched from these scrapings. By cultivation in serum free medium in non-adherent flasks these can be enriched in a few days (269). These cells grow in free-floating cell clusters (mammospheres), as have been described earlier. Cells without stem cell capabilities are not able to survive in the non-adherent, serum free conditions, and perish. This is a method of selecting cells with stem cell like capabilities according to their functionality, rather than surface markers.

3.3 PROTEIN EVALUATION

3.3.1 Immunohistochemistry and Immunocytochemistry

The proteins within the cell are vital for the biochemical reactions, structure and survival of the cell. The translation of different mRNA into proteins governs the function of that particular cell. In cancer, the regulation of the protein expression is often compromised. Over- or under-expression of different proteins is common in tumors. In oncological research it is therefore essential to investigate and compare the expression of different proteins within a tumor. Protein expression can be examined through immunohistochemistry (IHC) and immunocytochemistry (ICC) (270). IHC and ICC are methodically very similar, however IHC is performed on histological (tissue) sections whereas ICC is performed cytological (cell) material. Differences exist mainly in the preparation and fixation of the material. Both methods take advantage of the ability of the adaptive immune system to produce specific antibodies that bind to different proteins. This is then coupled to basic biochemical reactions to detect the presence of a specific protein. The cells of the adaptive immune system can be instructed to produce antibodies against any protein within the human cell. For research this

is done in two principally different ways. Either through injecting the targeted protein into an animal host where it will be identified as foreign and produce antibodies. The antibodies produced will target several structures of the protein of interest, yielding polyclonal antibodies. The second method instead uses immortalized B-cells *in vitro*. By introducing a gene fragment corresponding to a specific epitope into the B-cells, they start producing antibodies. These monoclonal antibodies target only one specific region of the protein of interest. The antibodies that are central in the IHC and ICC methods are also one of their main weaknesses. There is always a risk that the antibodies are not specific, but may also cross-react with other proteins, resulting in false positive results (271). Raising monoclonal antibodies against regions of the proteins that are unique can reduce this risk.

Several tests can be performed to examine the specificity of the antibodies, such as antigen blocking, where the antibody is saturated with antigen. Furthermore, one can also look for diminished staining of the antibody in a culture or tissue where the protein has been knocked out. Other biochemical methods, such as Western blotting, are commonly used for validation. If a protein is detected with the predicted molecular weight, this strengthens the evidence of the specificity of the antibody. An advantage of IHC is the additional spatial information of where or in which type of cells within a tumor a protein is expressed. This is not possible with other methods such as PCR or Western blotting. Therefore using IHC one can be more certain that the protein is present within the cells of interest, and not in the stroma or immune cells within the tumor.

One of the main challenges of IHC and ICC is how to reliantly quantify the staining of your protein of interest, especially when it is important to be able to compare the expression between different tumors. Examination of the sections has traditionally been by microscope and manual grading of the expression. The arbitrary grading system should also be suitable to the protein of interest. To reduce the observer bias and improve the reproducibility of the scoring, at least two independent observers often score the same samples. However, this is time consuming and do not completely remove observational bias. In the recent decade the increase in computer power has made it possible for digital image analysis programs to digitally quantify the expression of a protein. These programs have the potential to improve the reproducibility of the scoring significantly (272,273). Still, the image analysis software is not perfect in being able to distinguish all normal cells from cancer cells. Nor can it detect and count all cells within an area. The shape of a cancer cell can be very different, which sometimes makes it hard for the program to identify. Therefore, for some analysis programs there is a need to manually annotate the area of interest, e.g. the invasive cancer areas. Also it is important to review the detection rate of cells and examine the output of the image analysis software.

3.4 DNA AND RNA EVALUATION

3.4.1 Polymerase chain reaction

One of the most commonly used methods within molecular biology is the polymerase chain reaction (PCR) developed in the 1980's and later rewarded the a Nobel Prize in Chemistry (274). PCR is a fast and efficient method of amplifying and quantifying DNA and mRNA from different sources. Through repeated cycles, heat stable polymerases can copy a particular sequence of nucleotides *in vitro* in a short time span. The output DNA can then be used as input material in other biochemical methods. It can also be quantified to compare the relative or absolute amount of mRNA within a cell line. The main advantage of using PCR for the quantification of the expression of a gene is the robustness and reproducibility of the method. Using modern PCR with rapid thermal cycling and 96-well plates, a few hundred of samples or genes can be processed daily.

3.4.2 DNA Microarrays

The development of DNA microarrays before the turn of the last century started the era of global gene expression profiling. DNA microarrays are used to compare global changes in expression patterns between different tumors, but can also be used to compare differences in expression profiles with different treatments of cancer cells lines. The main principle of DNA microarrays is that a sample of DNA is allowed to hybridize to a single stranded complementary sequence of DNA corresponding to a gene transcript. The complementary DNA sequence is usually attached to a glass slide, depending on the manufacturer. Each potential gene transcript in the human cell has a corresponding complementary DNA sequence on the microarray chip. These DNA sequences are usually between 20 to 60 nucleotides long. If the gene is expressed or DNA present in a sample, it will hybridize to its complementary DNA sequence on the array and be detected. Detection is usually performed by fluorescently labeling the input DNA sample. Quantification is achieved since the intensity of the fluorescence is directly correlated to the number of mRNA molecules of that specific transcript in the input sample. Each complementary oligonucleotide is spotted to a specific location on the microarray. Thus, when scanning the fluorescently labeled array, each spot correlates to a specific transcript of a gene and each individual gene.

3.4.3 DNA sequencing

Since the discovery that DNA harbored the genetic code, many methods of DNA sequencing have been developed. In the last 10 years a rapid progress of the sequencing methods has taken place. This has resulted in an increase in speed, and at the same time, lowered the costs of sequencing. Modern massive parallel sequencing methods have revolutionized the research community and have also slowly begun to be introduced into the clinical setting. The sequencing is run in parallel to increases the speed (275). However, the reeds are shorter than traditional sequencing techniques (275). The exome, which encompasses the protein coding areas, only consists of only 1-2% of the complete human genome. Whole exome sequencing can therefore be more cost effective than sequencing of the whole genome (276).

Consequently, when only the exome is targeted, it can be sequenced with greater depth to uncover rare mutations within the tumors (277). During the preparation for exome sequencing, the exomes can be enriched using several different methods. Enrichment by PCR, ligation to magnetic beads, hybridization to complementary sequence or a combination of these is common. In study 4, we used a method from NimbleGen that uses hybridization to oligo-probes together with bead extraction.

To be able to reliably call the nucleotide variants that are present only in the tumor, so called somatic mutations, DNA from normal cells from the patients also need to be sequenced. The reason is to detect the germline nucleotide variants that the patient was born with. We used DNA extracted from blood samples from the patients. In parallel with the progress of sequencing techniques, several bioinformatics tools have been developed. These were specifically designed to aid in the analysis of sequence raw data. Since sequencing is performed in parallel, not from start to finish and on short sequences, reads are aligned against the reference genome. We used one of the most frequently used called Borrows-Wheeler Aligner (278). During the sequencing library preparation, PCR is performed on the input DNA yielding duplicate sequences. These are usually removed since they do not increase the information of the present mutations. Somatic variant callers can be used to compare the probability that a detected variant in the sequencing data is a true somatic mutation. There are several such tools to detect mutations within the tumors with high specificity and sensitivity. This means that they can reliably detect mutations with low allele frequency within the tumors (279). The accuracy of massive parallel sequencing is very good. However, the vast number of nucleotides examined means that there will be errors in the sequencing data (275). A way of decreasing the risk of false positives or false negatives is to increase the read depth. However, this will increase the cost. One method to confirm somatic mutations is to perform ultra-deep re-sequencing on some selected areas where the mutation has been found. Through improved depth, one can confirm or discard interesting mutations found in the first step of exome sequencing.

3.5 STATISTICS

Several statistical methods and tests have been performed to examine the hypotheses in our studies. For example, Student's T-test, Fisher's exact test, linear regression and survival analysis are a few of the methods used. The parametric Student´s T-test requires that the sample is normally distributed. If this assumption is not upheld, non-parametric tests such as the Mann-Whitney test can be performed.

In cancer research, identifying biomarkers that can be used to prognosticate the survival of the patients are important. In Sweden personal identification numbers and registries with high coverage can be cross-referenced. This makes up an important tool for researchers. Using the death registry or examining the electronic medical records of the patients, one can gather good quality data on follow-up. This can then be used to examine the prognostic effect of novel biomarkers on overall, disease-free or breast cancer-specific survival. In survival analysis, not only the event, e.g. death, but also the time until the occurrence of the event is

taken into consideration. This means that the effect of a prognostic marker to postpone the event can be studied. The Kaplan-Meier estimate can be used to estimate and visualize the survival probability. The Cox proportional hazard model is a statistical model to test survival differences between different groups of patients and can be used with both categorical and continuous explanatory variables. The Cox proportional hazard model yields the rate of an event per unit of time over baseline risk. Additional variables can be included in the model to control for these and reduce confounding. To withhold the assumptions of the model, enough events need to have taken place, this can sometimes be a problem when studying breast cancer patients since the survival is usually good, and if the cohort is not large enough the number of events can be too low. However, it has shown that only few events are needed if *a priori* hypothesis is present (280).

4 RESULTS AND DISCUSSION

4.1 PAPER I

"The dyslexia candidate gene DYX1C1 is a potential marker of poor prognosis in breast cancer"

Two studies published in 2009 implicated a role for DYX1C1 as a potential biomarker in breast cancer (195,196). However, the studies consisted of few patients and the authors were therefore unable to investigate the expression of DYX1C1 among different types of tumors or in association with prognosis. Prior these publications, very little was known of the role of DYX1C1 in cancer.

Thus, we examined the mRNA and protein expression of DYX1C1 in three independent breast cancer cohorts, in total 535 patients (Uppsala, Stockholm and CHARES cohort). The expression was examined against the clinicopathological characteristic and survival of the patients. Using data on mRNA expression from qPCR and microarray, we found that DYX1C1 was more highly expressed in ERα positive tumors. A similar association was seen between DYX1C1 and the progesterone receptor. DYX1C1 was also lower expressed in tumors of histological grade 3, compared to grade 1 and 2. Tumors of Basal and HER2-enriched intrinsic subtypes also had a lower expression than the Luminal A and B subtypes. Taken together, our results point towards an association of DYX1C1 mRNA expression with less aggressive tumor types.

When examining the protein expression of DYX1C1 using IHC, in tissue from 10 normal breasts we observed high expression of DYX1C1. A majority of the breast cancer tumors also expressed DYX1C1 protein. However, 11.3% of the invasive cancers had lost the expression. Using a univariate survival model, we also found that loss of DYX1C1 was associated with shorter overall patient survival. The association remained significant in a multivariate Cox regression model, after adjusting for age at diagnosis, lymph node status, grade, ERα- and PR-status. Women with tumors that were DYX1C1 negative had an increased risk of dying, with a hazard ratio of 3.44 (95% CI 1.84-6.42).

The knowledge of DYX1C1 in breast cancer is still limited. Today, only three studies, including ours have, been published that examines DYX1C1 as potential biomarker in the disease. The functional role of DYX1C1 in breast cancer is even less well studied. In dyslexia, DYX1C1 it is believed to be important in ER regulation, cilia formation and neuronal migration. Other genes associated to dyslexia such as DCDC2 and ROBO1 have been implicated in several cancers, perhaps indicating that this group of proteins has an important role in cancer physiology. DYX1C1 has been shown to regulate and be regulated by both ERα and ERβ. In fact, the promoter region and 5'-untranslated region of DYX1C1 contains several half-ERE and AP1-sites which ERs potentially can bind to under the right conditions (191). Although speculative, DYX1C1 may function as a regulator of the expression or degradation of ERs in breast cancer. Yet, with the limited knowledge about

DYX1C1 in breast cancers, it cannot be excluded that the gene is only an innocent bystander. Thus, further studies are needed to confirm DYX1C1's role in breast cancer.

4.2 PAPER II

"Low concordance of biomarkers in histopathological and cytological material from breast cancer"

The correct evaluation of biomarkers such as ERα, PR and Ki67 is essential for the correct treatment of breast cancer patients. We collected retrospective data from 346 patients of paired ICC and IHC evaluation (Immunochemistry concordance cohort). As a step in the diagnosis of breast cancer fine-needle aspirations (FNA) are taken from the tumors. These are then sometimes stained using ICC for several biomarkers. Although, it is not recommended that ICC should be used in the clinical decision making of the treatment of primary breast cancers, instead evaluation using IHC is considered gold standard. However, sometimes when examining metastatic lesions only FNA and thus ICC, may be available. Consequently, it is important that there is a high correlation between ICC and IHC.

Hence, we compared the correlation of ICC and IHC in ERα, PR and Ki67 evaluation using paired samples from the same tumor. In total we had data from both IHC and ICC on 133 patients for ERα, 80 patients for PR and 131 patients for Ki67. We found that on average, ICC evaluation of ERα expression was reported as 10.6 percentage points lower than when the same tumor was evaluated using IHC. When comparing positive from negative tumors using either 1% or 10% cut-off for ERα, 9.0% or 10.5% of the tumors, respectively, switched expression status from negative to positive or positive to negative. Similarly when evaluating PR, the expression was on average lower using ICC than IHC, this time by 13.6 percentage points. Using a 1% or 10% cut-off to discriminate positive from negative tumors, 7.5% and 11.3% of the tumors were differently classified, respectively.

When comparing the scoring of Ki67 expression, the expression was lower by 7.9 percentage points by ICC than IHC. Because there is no well-established cut-off for distinguishing between high and low proliferating tumors we used two different cut-offs; 14% and 20% which had been used by others previously (102). Using the 14% cut-off, 32.8% of the tumors changed proliferation classification from low to high or high to low. With a cut-off of 20%, 29.8% of the tumors were re-classified. We also showed that by adjusting the cut-offs used for ICC classification of Ki67 from 14% to 10% and from 20% to 14%, the number of patients that were re-classified was slightly decreased. Although, since the scoring of individual tumors could be either higher or lower in ICC compared to IHC, modifications of the cut-offs did not remove all the re-classification.

As a step in the routine diagnosis of breast cancer in Sweden, a FNA sample is often collected for analysis by a cytologist. Although, it is recommended that all predictive biomarkers should be analyzed on histological sections using IHC to ensure high validity, it is not unthinkable that ICC evaluations presented may influence decision-making at treatment

conferences. On the other hand, for metastatic lesions, ICC from FNA may perhaps be the only available source of material and is thus extremely important in the treatment decision.

We observed both over- and underestimation of ERα, PR and Ki67 when comparing ICC to IHC on individual cases, although on average, ICC systemically underclassified all three biomarkers. There could be several reasons for this, where one is the difference in the time point from when the samples were collected, where the FNA can precede the surgery by months. Another reason is that sampling using FNA represents a random small area of the tumor compared to the complete tumor evaluation by IHC. This is especially apparent for Ki67, since the recommendation is to evaluate the expression in the proliferative hot spots. Using ICC it is impossible to know if the sample has been extracted from the hot spot areas. In addition differences in fixation and preparation process are common between ICC and IHC and have been shown to cause variances in the intensity of staining. This is mainly believed to happen through differences in the deterioration rate of the biomarker.

When evaluating the data, we observed that a disproportional number of the ERα and PR grading was located around the cut-offs of 10%. This was seen in both the IHC and ICC evaluation, but was more common for ICC. The reason for this may be explained by a will of the cytologist and pathologist to not underdiagnose patients. This bias could be introduced unconsciously, to not withhold patients from endocrine treatment that is considered both efficient and safe. Additionally, it cannot be excluded that since the ICC evaluation is followed by an IHC evaluation, which the treatment should be based upon, the need for precision of the ICC evaluation decreases. This may also affect the accuracy of the ICC evaluation.

Using novel techniques of liquid and paraffin based ICC it has been shown that better concordance with IHC evaluation can be achieved. It is therefore important that pathological and cytological labs consider implementing these techniques. Evaluation of the correlation between ICC and IHC is important to decrease the variability of breast cancer scoring, especially for the diagnosis of metastatic lesions.

4.3 PAPER III

"Oestrogen receptor β1 and βcx have divergent roles in roles in breast cancer survival and lymph node metastasis"

The role of ERβ1 in breast cancer has been highly debated. We decided to investigate its prognostic significance and association to several clinical characteristics. We used a cohort of 340 women with local primary breast cancer and clinically negative axilla (The sentinel node cohort). We also examined the expression of ERα and ERβcx (ERβ2) in the same cohort. As described in other studies (281,282), we could observe that ERα was expressed predominantly in older women and in tumors of lower grade. ERβ1 was on the other hand equally expressed in patients of all ages and grades. ERβcx positive tumors showed a higher risk of having synchronous sentinel lymph node metastasis. However, no such association

was seen for either ERα or ERβ1. During the follow-up period, only 36 of the 340 patients died, whereof 16 deaths were due to breast cancer. Even with this few events, we found that ERα expression was associated to better breast cancer-specific survival. Also ERβ1 positivity was associated to both better breast cancer-specific and overall survival, this was not seen for ERβcx. The prognostic significance for ERβ1 was especially evident in high-grade tumors and in younger patients. Using a multivariable Cox regression model we found that ERβ1 remained an independent prognostic factor for both overall and breast cancer-specific survival. When examining the co-expression of ERα and ERβ1 in regards to the prognosis, the worst was seen in ERα and ERβ1 negative patients. Thus, the expression of either ERα or ERβ1 or both improved the prognosis.

There have been varied results among the studies that have examined the role of ERβ in breast cancer. The reasons for these could be several. One of them has been the lack of validated commercial antibodies, specific for different ERβ subtypes. Through the validation work by Dr. Valerie Speirs and her colleagues, the possibility to perform reproducible evaluation of ERβ in breast cancer tumors have greatly improved (283). In our study we used validated, commercially available antibodies. We also implemented digital image analysis software to reduce observer bias and increase the reproducibility of the scoring. Using histological sections instead of TMAs we could also reduce the effect of expression heterogeneity of the ERs within the tumors.

Our main finding, that ERβ1 seems to be an independent marker for good prognosis in breast cancer, is in accordance with most of the so far published studies (178-182). However, there are several studies that have failed to find such a connection (183,184). There is even one study that has shown ERβ1 to be a marker of poor prognosis (185). These discrepancies can perhaps be explained by differences described above when examining ERβ1 expression. Interestingly, we observed high expression ERβ1 and the strongest association to survival in the youngest patients and in patients with high-grade tumors. These are two groups that usually are considered to have worse prognosis. This may imply that ERβ1 could be used as a therapeutic target in these women. There are several compounds with selective affinity of ERβ1 over ERα that could be used as potential drugs to target ERβ1.

The splice variant ERβcx lacks the ability to bind ligand. It is instead thought to regulate the levels of ERα and ERβ1. In our study, we could not find any association of ERβcx with regard to the survival of the patients. Instead, high expression of ERβcx was associated with increased risk of synchronous lymph node metastasis. Since lymph node metastasis is considered one of the strongest prognostic risk factors, we would perhaps have observed a survival association for ERβcx in a larger cohort with more events.

The low number of events is also the main limitation of our study. We had 340 patients in our cohort, with a median follow-up for breast cancer-specific survival of almost 7 years and overall survival of more than 9.5 years. Still, total number of deaths limited the number of

sub-analysis that could be performed and the interpretation of these should therefore be viewed with some caution. However, we believe that our results may merit routine analysis of ERβ1 in breast cancer tumors, especially in younger women with high-grade tumors. This could perhaps improve the prognostic capabilities of the pathological examination in these patients. To examine if ERβ1 has a predictive role, a prospective study using ERβ1 as an indicator for endocrine treatment in a neoadjuvant setting might be promising.

4.4 PAPER IV

"Sequencing of breast cancer stem cell populations indicates a dynamic conversation between differentiation states in vivo"

A population of cells with stem cell capabilities can be identified in breast cancer tumors. They can be isolated either by their ability to form mammospheres or cell surface markers such as ALDH1, CD44 and CD24 (53,61,63). The origin and role of these cells in breast cancer tumors have been debated.

We set out to compare the mutational spectra of isolated breast cancer stem cells to that of the cells of the bulk primary tumor or non-stem cells. Our hypothesis was that breast cancer stem cells would harbor a higher frequency of the mutations that were most important in early tumor development and thus let us identify the so called driver mutations. We used whole exome sequencing and bioinformatics tools to identify somatic mutations. Mammosphere formation assays, and $ALDH1^{high}$ and $CD44^{+}/CD24^{-/low}$ isolation by FACS were used to identify breast cancer stem cells. We then compared, from the same tumor, the mutations found within the isolated stem cells to mutations found in the bulk tumor containing mostly differentiated cells. We also compared the stem cells to FACS sorted non-stem cells ($ALDH^{low}$ and $CD44^{-}/CD24^{+/-}$). The combination of these two are from here on called the "bulk tumor/non-stem cells".

Contradictory to our hypothesis, the mutations discovered were present in both the stem cells and the bulk tumor/non-stem cells at the same frequency. Irrespective of the isolation method (mammosphere of FACS) we could show similar overlap of somatic mutations in the stem cells and bulk tumor/non-stem cells. Interestingly, the allele frequency of the mutations was highly correlated between the stem cells and the bulk tumor/non-stem cells.

However, a few mutations were unique to either the stem cells or cells of the bulk tumor/non-stem cells; these were often of low allele frequency. To invastigate that the unique mutations were not sequencing errors or missed due to low coverage, we selected 14 mutations sites in three different patients and performed ultra-deep amplicon-sequencing. These were either unique or shared mutations present in the stem cells or bulk tumor/non-stem cells. This allowed us to validate that three of the shared mutations in the original sequencing remained shared also using the ultra deep sequencing. The 11 mutations that were either unique to the cancer stem cells or to the bulk tumor/non-stem cell were either false positive (four mutations), technical artifacts (one mutation) or shared (five mutations). However, one

mutation was confirmed to be unique in the cancer stem cells, although at a low allele frequency of 5%. Thus, these could originate from a local clone of cells.

Our results support the theory that breast cancer stem cells are a dynamic phenotype present in breast cancer tumors. We could not find evidence that breast cancer stem cells give rise to the rest of the tumor through a hierarchal model. If this were the case fewer mutations should be found in the stem cells and some mutations should be unique to the bulk tumor/non-stem cells. The most complete way of explaining the presence of the same mutations at equal allele frequencies in both stem cells and non-stem cell compartments, is by a dynamic transition model. This suggests that cells with differentiated phenotype can transform into cells of stem cell phenotype and vice versa.

In one study, no differences were seen in copy number variations of $CD44^+$ cells compared to $CD24^+$ in breast cancer cells (284). In a study by Gupta et al., cell lines from Luminal, Basal or stem cell subtype origin showed striking transforming capabilities into the other subtypes after cultivation (67). This suggests that differentiated breast cancer cells have the capabilities to transform into both stem cells and cells of other subtypes. In breast tissue, where the sheading of epithelial cells occurs at slower rates, there is more time for differentiated cells to become malignant. Therefore all cancer cells do not have to originate from the stem cells. This may be the case for breast cancer, where genomic events perhaps can result in a differentiated cell to acquire stem cell capabilities. A hierarchal stem cell model may be more suitable in cancers were the turn-over rate of the cells are higher, for example in the gut or in the bone marrow. In these tissues the time to obtain the vital genomic events to become malignant is too short and therefore only can take place in the long-lived stem cells.

The mechanisms of dynamic stem cell formation have been suggested to be similar to that of epithelial-to-mesenchymal transition (EMT). EMT is thought to occur due to epigenetic changes, especially DNA hypomethylation (64). Breast cancer stem cells have shown to harbor hypomethylation of several transcription factors involved in EMT. Silencing of micro-RNAs through hypermethylation has also been shown in both EMT and breast cancer stem cells (64). Furthermore, overexpression of the transcription factors Twist and Snail, important in EMT, in immortalized human mammary epithelial cells increased the mammosphere formation (285). In addition treatment using TGFβ have shown to produce both EMT and breast stem cells (69).

4.5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The treatment of breast cancer has improved drastically in the last 100 years. Today, most women who are diagnosed with breast cancer have a good prognosis. However, due to the high incidence many women still die of the disease. The increased life expectancy and changes in life style means that the incidence of many cancers, among them breast cancer have slowly increased. As pointed out by Bert Vogelstein in a recent review, bad luck is one of the major risk factors for developing cancer (286). Therefore, complete prevention or eradication of all cancers will probably not be possible. Instead, early detection and further

improving the treatment will be increasingly important. Identification of novel biomarkers and understanding the biological processes of breast cancer development are important early steps in later improving the survival. Both ERβ1 and DYX1C1 have the potential to become important breast cancer biomarkers or lead to the identification of novel mechanism of breast cancer development. In the end they could perhaps become novel targets for treatment. ERβ1 is especially interesting since it is a receptor and therefore easily can be targeted by selective drugs. Whereas studying DYX1C1 may instead lead to better understanding of the properties needed for cellular migration and ER regulation. Due to the inconsistencies observed in the effect of ERβ1 on survival, the role of ERβ1 in breast cancer is not clearly understood. However, the majority of *in vitro* and *in vivo* studies points towards a protective role (167). The main goal would be to test to treat breast cancer patients with ERβ1 targeting drugs. Still today we are far from that goal, since the scientific basis of ERβ1 role in breast cancer needs to be strengthen and also the treatment needs to be tested in animal models.

Fine needle aspirations are a safe and cost efficient method of identifying malignant cells. It is also sometimes the only available method of extracting cells from distant metastasis for the analysis of biomarkers. This is especially important since studies have shown that the status of several biomarker can switch from primary tumor to metastasis lesions (287). Nevertheless, it is important for clinicians to understand that results from ICC and IHC are not always equivalent, suggesting that some tumors can be wrongly classified as either ERα negative or positive, thus resulting in the wrong treatment being given. Instead of performing analysis of FNA using ICC, quantification of ERα by RNA-sequencing could perhaps increase the reproducibility. We are presently part of a project that intends to evaluate the accuracy and efficiency of RNA-sequencing compared to IHC for several of the routine biomarkers. Perhaps this can be done for FNA of metastatic lesions as well.

The role and presence of cancer stem cells in breast cancer are far from settled. Today most would agree that there is a subset of breast cancer cells that have increased malignant potential. If these should be called stem cells or not is perhaps more a case of semantics. Finding a way to target these cells, which seem to have higher resistance to common chemotherapy and radiotherapy will become important. The understanding that breast cancer stem cells is a phenotypic state rather than a fixed subset of cells leads to the consequence that new breast cancer stem cells can be generated from even a small number of differentiated breast cancer cells. These cells could therefore result in a relapse if these cells are not sufficiently targeted. Hence, finding treatments that are efficiently targeting both subsets of cells could perhaps be beneficial for the patients and improve survival. One of the main difficulties when studying breast cancer stem cells is the lack of a universal biomarker. The identification of such a biomarker would greatly improve the understanding of breast cancer stem cells and would be important for the future studies and understanding of these.

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